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GENETIC AND MORPHOLOGICAL STUDIES OF TUMOR HETEROGENEITY, MULTIFOCALITY AND OUTCOME IN LOCALLY ADVANCED PROSTATE CANCER

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Genetic and morphological studies of tumor heterogeneity, multifocality and outcome in locally advanced prostate cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Prostate cancer is a leading cause of mortality worldwide. Patients with locally advanced disease have a higher risk of relapse after treatment with curative intent. Examples of risk factors are seminal vesicle invasion (SVI) and lymph node metastases. However, not all patients with adverse pathology experience recurrence. It is not fully understood how histopathological features are associated with progressive capacity and how to best identify them before treatment. The aim of this thesis was to evaluate histopathological and genetic prognostic factors of locally advanced prostate cancer and to correlate findings in preoperative biopsy with radical prostatectomy (RP) specimens.

In **Study I** we evaluated 1050 RP specimens. SVI was found in 60 cases, which were further analyzed regarding pathological factors such as route of invasion, tumor area and Gleason score of the cancer component invading the seminal vesicles. We confirmed that patients with SVI have a higher risk of biochemical recurrence. The prognosis was poorer if cancer invaded the mucosa of the seminal vesicle compared to when cancer invasion was restricted to the muscular wall.

The aim of **Study II** was to evaluate 45 morphologically distinct tissue areas in a RP specimen with lymph node metastases in order to identify the clone that gave rise to the metastases. We analyzed break-point regions, which marks a start of an amplification or a deletion event, to construct a phylogenetic tree showing the somatic relationship between samples. The greatest similarity with metastases was seen in three samples with intraductal carcinoma. This lesion has previously been associated with poor prognosis, although this study was the first to indicate a metastatic potential.

In **Study III** we analyzed the prognosis of patients with SVI compared to patients with extraprostatic extension alone (stage categories pT3b and pT3a, respectively). Data from 4063 pT3a cases and 1371 pT3b cases were retrieved from the National Prostate Cancer Register. We found that patients with stage category pT3b had a higher risk of death from prostate cancer and were more likely to receive postoperative treatment with androgen deprivation therapy or radiotherapy. They also had a greater tumor burden as measured by tumor length and number of positive cores and a higher Gleason score.

In **Study IV** we evaluated several tumor foci and biopsy cores from 11 patients. The samples were sequenced and somatic profiles of the tumor foci compared with those of the biopsies. We found a high degree of genomic heterogeneity between foci within the same prostate. In eight patients the biopsies represented at least one of the tumor foci of the RP specimen. In only one case two somatically distinct tumors were identified in the biopsies.

In conclusion, these studies show that prostate cancer is a morphologically and genetically heterogeneous disease. The poor representation of somatically different tumors in core biopsies suggests a diagnostic challenge as we move towards more individualized treatment.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är den vanligaste cancertypen i Sverige med ca 10 000 nya fall varje år. Lokaliserad prostatacancer kan botas antingen genom strålbehandling eller kirurgi. När prostatacancer växer utanför prostatakapseln eller det finns spridning till lokala lymfkörtlar säger man att tumören är lokalt avancerad. Dessa tumörer är svårare att behandla och det finns en högre risk för att sjukdomen kommer tillbaka efter försök till bot. Exakt vilka egenskaper hos tumören som gör att den kan sprida sig utanför prostatan är inte känt. Detta är viktigt att kartlägga då det kan förändra hur vi behandlar dessa tumörer.

Sädesblåsorna består av två avlånga körtlar som ligger vid bakre övre hörnet av prostatan. När cancer invaderar sädesblåsan vilket även kallas för vesikelinvasion, är prognosen sämre för patienten. Sådan typ av tumörväxt kan vara svårt att diagnostisera före behandling och det är oftast först när patologen tittar på den bortopererade prostatan i mikroskop som man säkert kan ställa diagnos. Det har dock visat sig att vissa patienter med vesikelinvasion har god prognos. I Studie I och III i denna avhandling utvärderades prognosen för patienter med vesikelinvasion. I Studie I analyserade vi totalt 1050 bortopererade prostator i mikroskop, varav 60 hade vesikelinvasion. Dessa fall utvärderades med avseende på ett antal olika faktorer såsom spridningssätt, tumörarea, tumörgrad och lateralitet. Vi kom fram till att i de fall då tumören växte in i slemhinnan hade patienterna en högre risk för återfall. Detta har aldrig tidigare rapporterats. Patienterna som endast hade tumörinvasion i muskelväggen av sädesblåsan hade en lika god prognos som patienter utan vesikelinvasion. I studie III använde vi registerdata och analyserade 1371 fall av vesikelinvasion och 4063 fall av tumörgenombrott av prostatakapseln som inte involverade sädesblåsan. Vi fann att patienter med vesikelinvasion har en sämre prognos jämfört med patienter som har tumörgenombrott av prostatakapseln utan vesikelinvasion. De hade en högre risk att få tilläggsbehandling efter operation (hormoner och strålbehandling) och även en högre risk att dö av prostatacancer. Vidare undersökte vi de diagnostiska biopsierna som tagits före operationen och såg att patienter med vesikelinvasion hade en högre tumörbörda i biopsierna med avseende på tumörlängd, antal biopsier med tumör och grad.

I Studie II undersökte vi ett fall där prostatacancer spridit sig till regionala lymfkörtlar i bäckenet. Vi mikrodissékerade 45 morfologiskt olika områden i prostatan i ett laserdissektionsmikroskop. Vi undersökte DNA-förändringar i de olika områdena och jämförde med de förändringar som fanns i metastaserna. Tre områden visade stor somatisk likhet med metastaserna och det visade sig att alla dessa hade samma morfologi - intraduktal cancer. Denna typ har tidigare associerats med dålig prognos men man vet inte riktigt varför. Denna studie var först med att visa att de somatiska förändringarna som finns i intraduktal cancer även finns i metastaserna vilket inger misstanke om att intraduktal cancer har potential att metastasera. Därför är det särskilt viktigt att rapportera fynd av intraduktal cancer om man hittar det i diagnostiska biopsier.

I Studie IV undersöktes 11 fall av multifokal prostatacancer samt tillhörande diagnostiska biopsier. Syftet var att undersöka hur väl biopsierna representerar de olika tumörområdena

som finns inom en prostata ur ett genetiskt perspektiv. Detta har betydelse då vi i framtiden sannolikt kommer att behandla tumörer olika beroende på vilka somatiska förändringar tumören bär på. I endast ett av fallen hittade vi somatiska förändringar i biopsierna som stämde överens med förändringarna inom de två stora tumörområden vi fann i prostatan. Denna studie tyder på att diagnostiska biopsier ger en otillräcklig bild av de genetiskt heterogena tumörerna som kan finnas inom en och samma prostata. Vidare kom vi fram till att det är en stor heterogenitet i genetik mellan olika tumörområden inom samma prostata. Detta innebär utmaningar i den kliniska vardagen då man framöver hoppas kunna förbättra behandlingen baserat på genetisk analys av tumörvävnad före operation.

Sammanfattningsvis visar dessa studier att prostatacancer är mikroskopiskt och genetiskt en mycket heterogen sjukdom. Idag känner vi till flera olika riskfaktorer som innebär en högre risk för återfall i sjukdom för patienten. Studierna i denna avhandling har dels bekräftat tidigare riskfaktorer, dels påvisat nya riskfaktorer, såsom slemhinneinvasion i sädesblåsan. Vidare har vi för första gången visat ett samband mellan intraduktal cancer och metastaserande potential. Den genetiskt heterogena naturen i prostatacancer leder till utmaningar i diagnostiken och vi ser att representationen av dessa olika tumörer är låg i de diagnostiska biopsierna. Detta innebär utmaningar ur ett kliniskt perspektiv där vi går mot ett alltmer individanpassat behandlingsupplägg.

LIST OF SCIENTIFIC PAPERS

- I. **Kristiansen A**, Wiklund F, Wiklund P, Egevad L.
Prognostic significance of patterns of seminal vesicle invasion in prostate cancer
Histopathology. 2013 Jun;62(7):1049-56
- II. Lindberg J*, **Kristiansen A***, Wiklund P, Grönberg H, Egevad L.
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- III. **Kristiansen A**, Drevin L, Delahunt B, Samaratunga H, Robinson D, Franck Lissbrant I, Stattin P, Egevad L.
Prognostic significance and biopsy characteristics of prostate cancer with seminal vesicle invasion on radical prostatectomy: a nationwide population-based study
Pathology. 2017 Dec;49(7):715-720
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Somatic alterations detected in diagnostic prostate biopsies provide an inadequate representation of multi-focal prostate cancer
The Prostate. 2019;1-9

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LIST OF RELATED PUBLICATIONS

Miyai K, **Kristiansen A**, Egevad L, Pina-Oviedo S, Divatia MK, Shen SS, Miles BJ, Ayala AG, Park YW, Ro JY.

Seminal vesicle intraepithelial involvement by prostate cancer: putative mechanism and clinicopathological significance

Hum Pathol. 2014 Sep;45(9):1805-12

Stinnesbeck M*, **Kristiansen A***, Ellinger J, Hauser S, Egevad L, Tolkach Y, Kristiansen G.

Prognostic Role of TSPAN1, KIAA1324 and ESRP1 in Prostate Cancer

Submitted

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LIST OF ABBREVIATIONS

ADT	Androgen deprivation therapy
AMACR	Alpha-methylacyl-CoA-racemase
AR	Androgen receptor
BPR	Break-point region
CNV	Copy number variation
DRE	Digital rectal examination
EPE	Extraprostatic extension
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescent in situ hybridization
GS	Gleason score
H&E	Hematoxylin and eosin
IDC-P	Intraductal carcinoma of the prostate
LCM	Laser capture microdissection
LOH	Loss of heterozygosity
NPCR	National Prostate Cancer Register
PSA	Prostate-specific antigen
PZ	Peripheral zone
RP	Radical prostatectomy
RT	Radiotherapy
SNP	Single nucleotide polymorphism
s-PSA	Serum prostate-specific antigen
SVI	Seminal vesicle invasion
TNM	Tumor, node, metastasis
TZ	Transition zone

1 INTRODUCTION

1.1 EPIDEMIOLOGY

Prostate cancer is a leading cause of morbidity and mortality worldwide and the second most common cancer among men (1). In Sweden, it is the most common non-cutaneous malignancy where it accounts for approximately one third of all cancers (2). The incidence of prostate cancer has increased rapidly during the last decades as a result of serum prostate-specific antigen (s-PSA) testing and increased life expectancy (3). In 2016, 10 473 men were diagnosed with prostate cancer and 2 347 men died of the disease in Sweden (2). It becomes increasingly common with higher age. In 2016, the median age at diagnosis was 70 years, however, 151 men were younger than 50. Globally, almost 1.3 million new cases and over 350 000 prostate cancer associated deaths were estimated in 2018, ranking as the fifth leading cause of cancer death in men (1). The incidence rate is high in Australia/New Zealand, Northern and Western Europe and North America (Figure 1). The varying incidence of prostate cancer between regions is partly explained by differences in diagnostic opportunities, including both s-PSA testing and core biopsies. However, the incidence is highest among men of African descent in the United States and the Caribbean, which reflects a genetic predisposition (4). In contrast, prostate cancer is less common in Asia. Studies have shown that the incidence increases in men who migrate from low to high incidence areas (5, 6). For example, Japanese men who migrate to the United States have a higher risk of being diagnosed with prostate cancer compared to men who remain in Japan (5).

One of the unique features of prostate cancer is that many men live with latent, clinically undetectable tumors. In autopsy studies histologic prostate cancer is usually found in 20-30% of cases (7, 8). In a classic study by Sakr *et al*, latent prostate cancer was found in 64% of men aged 60 to 69 years (8). In a Hungarian study, prostate cancer was seen in 38.8% of 139 autopsy cases between 18 and 95 years (9). However, in the age group 81 to 95, the prevalence was as high as 86.6%. Haas *et al* examined 164 autopsy cases and found prostate cancer in 29% but only 42.6% were considered clinically significant (7). As indicated by these autopsy studies, many men have indolent, clinically undetected tumors. Thus, one of the great challenges in the care of prostate cancer is to distinguish patients with aggressive disease who need early curative treatment from those with indolent tumors that can be monitored.

Estimated age-standardized incidence rates (World) in 2018, prostate, males, all ages

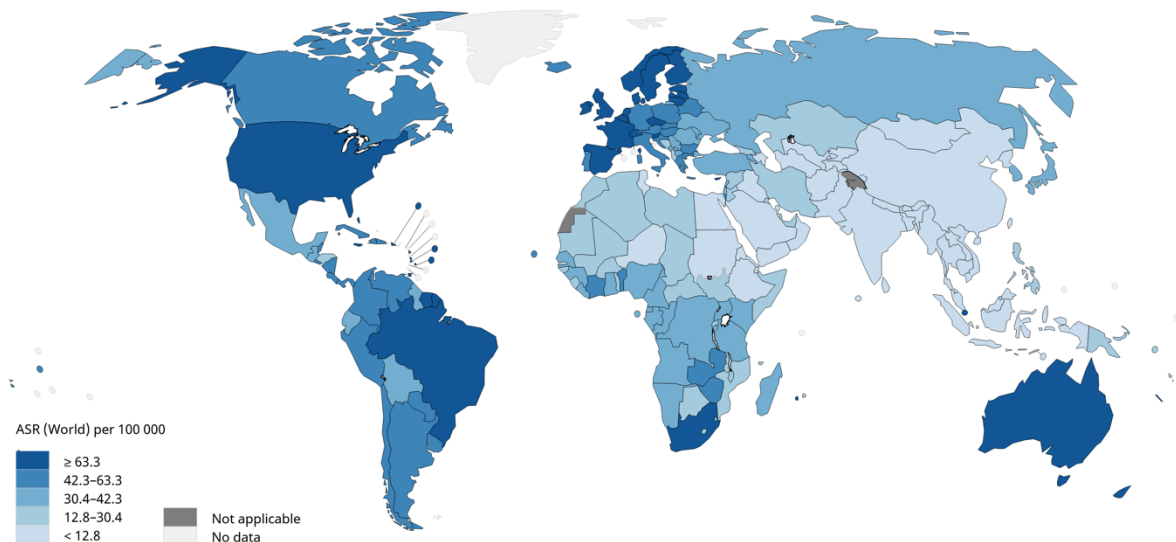


Figure 1. The incidence rate of prostate cancer worldwide in 2018. The incidence is high In Western countries. Reprinted with permission from <http://gco.iarc.fr/today>

1.2 RISK FACTORS

Although the etiology of prostate cancer is not yet completely understood, there are a few well-established risk factors including age and place of birth. Migration studies support the theory of prostate cancer as a multifactorial disease involving, not only genetic factors, but also lifestyle and environmental factors. A few studies have investigated the association between diet and prostate cancer. Dietary fat, red meat, Vitamin E, selenium and tomatoes are examples of nutrients that have been suggested to either increase or reduce the risk of prostate cancer. High intake of dietary fat has been suggested to increase the levels of circulating androgens and thereby increase the risk of prostate cancer (10). Red and processed meat have also been associated with an elevated risk of prostate cancer (11). By contrast, daily intake of tomatoes and tomato products seems to reduce the risk (12).

Family history plays an important role with an approximately two- to three-fold increased risk for men with affected first-degree relatives (father, brother, son) (13). The risk generally increases with the number of affected relatives. Certain genetic traits have been associated with increased risk of developing prostate cancer. It is estimated that around 5% of prostate cancers develop from mutations in highly penetrant cancer predisposition genes, such as *HOXB13*, *BRCA1* and *BRCA2* (14, 15). *BRCA1* and *BRCA2* are tumor suppressor genes and mutations in these genes contribute to a higher risk of early onset of prostate cancer and worse prognosis (16, 17). Studies have shown that *BRCA2* carriers have a higher risk

compared to *BRCA1* carriers (18, 19). Patients with *BRCA1* and *BRCA2* mutations and metastatic prostate cancer have recently been shown to have a high response rate to the PARP-inhibitor olaparib (20). Although there are a few germline variants that are associated with high risk of prostate cancer, the disease more commonly develops as a result of a combination of genetic changes and environmental factors or sporadic somatic mutations.

Genome wide association studies have helped to identify single nucleotide polymorphisms (SNPs) associated with prostate cancer. While each SNP alone is associated with a rather small risk, combinations of several SNPs may increase the risk significantly. More than 200 SNPs have been identified, which are estimated to account for around 30% of inherited risk of prostate cancer (21, 22).

1.3 CLINICAL FEATURES AND DIAGNOSIS

1.3.1 Clinical features

Men with early stage prostate cancer are commonly asymptomatic. As the disease progresses the patient may present with lower urinary tract symptoms, such as increased frequency, weak stream, nocturia and urgency. Patients with these symptoms often have locally advanced disease, which may or may not be curable. Prostate cancer often metastasizes to lymph nodes and bone. Liver and lung metastases also occur, although more rarely. Patients with advanced disease may present with bone pain, loss of weight and fatigue. S-PSA testing combined with digital rectal examination (DRE) are included in the initial diagnostic work-up for prostate cancer. If these tests detect an abnormality, the patient is usually further examined with ultrasound, core biopsy and magnetic resonance imaging (MRI).

1.3.2 DRE

DRE is a fast, safe and cost-effective diagnostic tool and therefore usually recommended as routine examination in the diagnostic process of prostate cancer. However, it is dependent on the examiner and the result should therefore be interpreted cautiously (23). In a recent systematic review the sensitivity and specificity for DRE for prediction of prostate cancer in symptomatic patients was 28.6% and 90.7%, respectively (24). The relatively low sensitivity indicates that many patients diagnosed with prostate cancer do not have an abnormal DRE. Therefore, patients with suspected prostate cancer should be further investigated regardless of the DRE result.

1.3.3 PSA

Prostate-specific antigen (PSA) is an enzyme that is produced in the epithelial cells of the prostate and secreted into the prostatic ducts. A small amount of PSA enters the circulation. In some conditions, including cancer and prostatitis, the basement membrane of the epithelial cells is disrupted, which leads to higher levels of PSA in the blood. Therefore, s-PSA is usually higher in men with prostate cancer than in those without cancer, although cancer cells usually produce less PSA compared to normal epithelial cells. Poorly differentiated prostate cancer cells may entirely lose the ability to express PSA and these tumors are often clinically aggressive. S-PSA testing was clinically introduced in the 1980s (25), however, until the early 1990s it was mostly used for monitoring treatment response in patients with advanced prostate cancer.

1.3.4 MRI

MRI has become increasingly important in the diagnostic process of prostate cancer. Its functions include identification, risk stratification and staging of tumors and improved biopsy sampling through MRI guidance. The ability to detect clinically significant tumors through multiparametric MRI has improved during the last decade (26). The images are evaluated by a radiologist and the level of suspicion of prostate cancer is reported according to the PI-RADS (Prostate Imaging Reporting and Data System). Furthermore, using MRI-targeted biopsies helps to identify and sample suspicious areas of the prostate. Studies have shown that when patients undergo MRI followed by targeted biopsies, fewer patients are biopsied and more clinically significant cancers are detected (27, 28).

1.3.5 Core biopsies

Men with elevated s-PSA are usually further investigated with 8-12 core biopsies, which are either targeted or systematic. It is most commonly guided by transrectal ultrasound, although MRI targeted biopsies may improve sampling of the clinically most important tumor. The biopsies are graded according to the Gleason grading system, which is an important tool in the treatment planning process. In addition to Gleason score (GS), the pathology report includes number of cores with cancer and cancer length in mm. Extraprostatic extension (EPE), seminal vesicle invasion (SVI) and lymphovascular invasion should also be reported if present. Biopsies only sample a small fraction of the prostate and may not be representative of the overall morphology. Therefore, undergrading of the tumor sometimes occurs. At the 2014 ISUP consensus conference the grading of prostate cancer was updated (29), which has led to better agreement between biopsy and radical prostatectomy (RP) score (30).

1.3.6 Liquid biopsies

Blood samples, containing tumor biomarkers, so called liquid biopsies, could potentially remedy some of the problems with representative sampling. If the most aggressive cancer has the highest fraction in blood, it may aid multifocal profiling in deciding which genomic profile that should be applied for prognostication. However, future studies have to be conducted to demonstrate if this is possible or not. Therefore, liquid biopsies may have an increasingly important role in the early detection, prognostication and treatment planning of prostate cancer. However, in patients with localized disease circulating tumor cells and DNA are detected at very low levels (31, 32). Therefore, to perform comprehensive genomic profiling, needle biopsies of the prostate will remain an important source to access the alterations of the tumor genome.

1.3.7 The dilemma of prostate cancer diagnostics

In Sweden, opportunistic s-PSA-screening has been applied in recent decades. This means that men have s-PSA tests on their own initiative. Since the clinical introduction of s-PSA testing, more asymptomatic patients have been diagnosed with prostate cancer. A consequence of increased diagnostic activity is that more patients are diagnosed with relatively well-differentiated, clinically insignificant tumors. Treatment with curative intent, radiotherapy (RT) or RP, is related to side effects such as urinary incontinence and impotence. Men who otherwise would never have been diagnosed with symptomatic prostate cancer risk undergoing treatments with lifelong side effects that often impact their quality of life. Overdiagnosis and subsequent overtreatment of indolent prostate cancer is therefore a great concern. S-PSA has a high false positive rate and an elevated value may be seen in a number of conditions other than prostate cancer, such as benign prostatic hyperplasia, urinary tract infection and prostatitis. Patients with elevated s-PSA usually undergo core biopsies but this procedure is associated with complications, most commonly infections (33). Due to these issues, s-PSA-screening in asymptomatic patients has been considerably debated. Several studies have been carried out with the purpose to improve the diagnostic process of prostate cancer. The STHLM3 study (34), which was published in 2015, evaluated a new model for detection of prostate cancer. The test is a combination of plasma protein markers, genetic polymorphisms (232 SNPs), and clinical variables. The model was tested in men aged 50-69 in the Stockholm region and identified clinically significant high-risk cancers, defined as cancers with GS of at least 7, with greater specificity than s-PSA alone. As for MRI targeted biopsies, this test could reduce unnecessary biopsies, which is highly valuable in a screening situation. In an ongoing study the combination of STHLM3 test with MRI and following

targeted biopsies is evaluated (35). This model is suggested to further improve the specificity in prostate cancer detection and reduce the risk of overdiagnosis.

1.4 TUMOR CLASSIFICATION

1.4.1 Localization and histological subtypes

Prostate cancers are usually multifocal with at least 2-3 tumor foci of different histological architecture (36, 37). Arora *et al* found two or more cancer foci in 87% of 115 whole-mount prostatectomy specimens (36). In these foci, the GSs were commonly different from the overall score of the tumors. The majority of tumors arise in the peripheral zone (PZ) of the prostate, but some arise in the transition zone (TZ). Central zone tumors are rarely seen unless it is secondarily involved. More than 90% of all primary prostatic tumors are acinar adenocarcinomas. Ductal adenocarcinoma, which is often seen in combination with acinar adenocarcinoma, is the second most common histologic subtype and associated with poor prognosis (38). Other variants of prostatic carcinoma are uncommon and include basal cell carcinoma, neuroendocrine tumors and sarcomatoid carcinoma.

1.4.1.1 Intraductal carcinoma of the prostate

Intraductal carcinoma of the prostate (IDC-P) is a lumen-spanning proliferation of prostate adenocarcinoma cells within pre-existing benign ducts and acini (Figure 2). IDC-P has a basal cell layer that is at least focally preserved. It is seen in about 20% of RP specimens and almost always together with invasive adenocarcinoma (39). IDC-P without an invasive component of adenocarcinoma has only been reported in a few cases (40). Therefore, curative treatment is often recommended for men with IDC-P on needle biopsies, even in the absence of invasive carcinoma. It has been shown that IDC-P shares several somatic alterations with cribriform invasive cancer, suggesting a close association between this feature and invasive prostatic carcinoma Gleason grade 4 and 5 (41). However, the International Society of Urological Pathology (ISUP) consensus conference in 2014 recommended that IDC-P should not be graded (29). A survey study showed that the reporting of IDC-P varies among European uropathologists, suggesting that standardization of reporting is needed (42). IDC-P is associated with advanced tumors and studies have demonstrated that patients with components of IDC-P have a poorer clinical outcome (43-45).

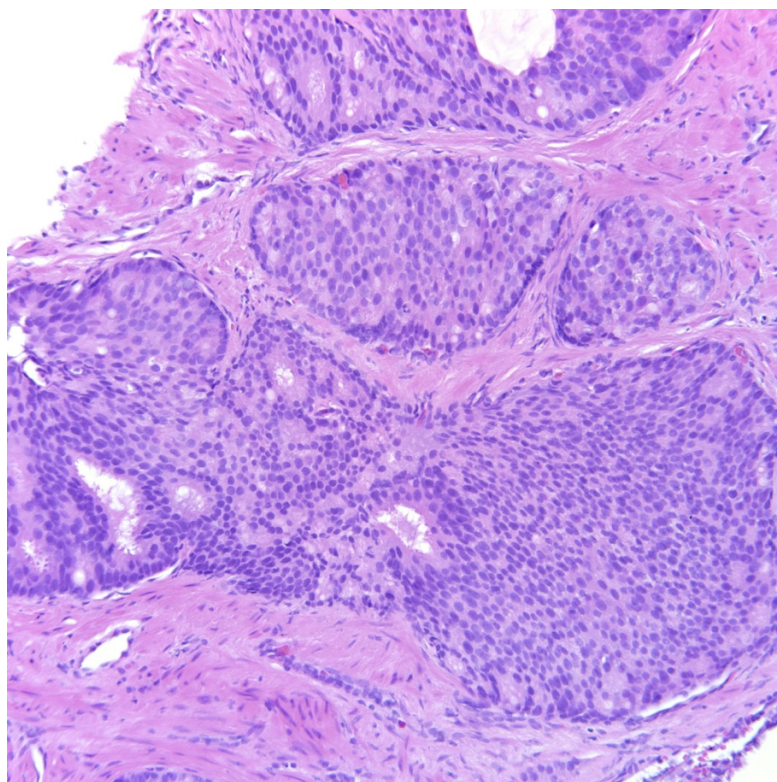


Figure 2. Histologic slide showing intraductal carcinoma of the prostate. Prostate cancer cells with markedly enlarged nuclei filling up the lumen of prostatic ducts.

1.4.2 Gleason grading system

Morphological grading is an important prognostic factor in tumor pathology. The Gleason grading system was established by Donald Gleason in the 1960s (46) and is the most widely used grading system for prostate cancer. The grading scale is based entirely on the architectural patterns of the prostatic glands. The five basic growth patterns range from 1 to 5, where 1 is the most differentiated and 5 the least differentiated (Figure 3). Adding the primary grade (the dominant pattern of the tumor) and the secondary grade (the second most frequent pattern) generates the GS. Tumors with higher GS are more aggressive and associated with a worse outcome. Over the last decade there has been a significant inflation in Gleason grading (47). The system was revised at the ISUP consensus conference in 2005 and further revised at another consensus conference in 2014 (29, 48). Morphological criteria were updated including the definition of Gleason pattern 4. The use of Gleason patterns 1 and 2 is no longer recommended since they do not seem to indicate an outcome different from that of Gleason pattern 3 cancers. In addition it was suggested that the GSs should be grouped in ISUP grades 1 (GS 2-6), 2 (GS 3+4=7), 3 (GS 4+3=7), 4 (GS 8) and 5 (GS 9-10). These

groups predict prognosis and a scale ranging from 1-5 is also more intuitive for the patients (49, 50).

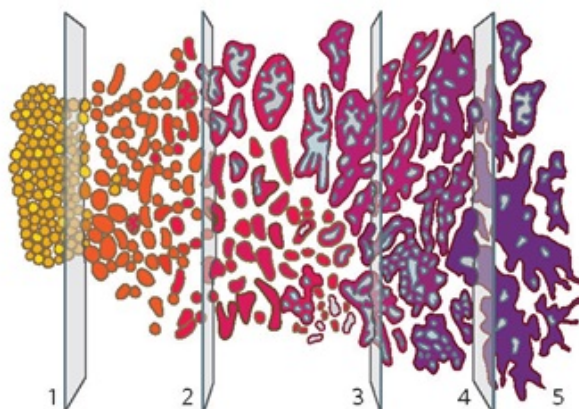


Figure 3. Cancer cells and gland architecture according to the Gleason grading system with grades from 1 (highest differentiation) to 5 (lowest differentiation). Reprinted with permission from www.prostate.org.au

1.4.3 TNM-classification

The most widely used staging system for prostate cancer is the American Joint Committee on Cancer (AJCC) tumor, node, metastasis (TNM) system (Table 1). The last edition (8th edition) was published in 2018 (51). The TNM stage combined with GS and s-PSA, gives an estimate of prognosis and is considered the basis for guiding of treatment decisions in prostate cancer patients (52). In addition, the TNM staging system is an important tool in clinical research and enables objective comparisons between clinics and countries.

Table 1. TNM classification of prostate cancer according to the AJCC TNM system

Category	Criteria
<i>T (primary tumor)</i>	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor that is not palpable
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy found in one or both sides, but not palpable
T2	Organ confined
T2a	Tumor involves one half of one side or less
T2b	Tumor involves more that one half of one side but not both sides
T2c	Tumor involves both sides

T3	Extraprostatic extension
T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
<i>N (regional lymph nodes)</i>	
NX	Regional lymph nodes were not assessed
N0	No positive regional lymph nodes
N1	Metastases in regional lymph nodes
<i>M (distant metastasis)</i>	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

1.5 PROGNOSIS AND PROGNOSTIC FACTORS

The prognosis of men with prostate cancer varies significantly. The relative 5- and 10-year survival of patients with prostate cancer in Sweden is 93% and 88%, respectively (2).

Currently, many men are diagnosed with low-risk disease, which has little impact on survival. Patients with clinically localized disease are usually stratified into three prognostic subgroups. This model predicts the risk of biochemical failure after curative treatment based on clinical stage, s-PSA and GS. Patients are classified as follows:

- Low-risk: Stage \leq T2a and s-PSA level \leq 10 ng/ml and GS \leq 6
- Intermediate-risk: Stage T2b or s-PSA level $>$ 10 and \leq 20 ng/ml or GS 7
- High-risk: Stage \geq T2c or s-PSA level $>$ 20 ng/ml or GS \geq 8

A large study based on data from the National Prostate Cancer Register (NPCR) reported a 10-year mortality for non-curatively treated low- and high-risk prostate cancer, ranging from under 5% to almost 30%, respectively (53). The median survival for patients with distant metastases at diagnosis was previously around 2.5 years (54). However, in recent years many new treatments have been introduced for these patients and the median survival has increased accordingly. Patients with bone metastases have a better prognosis than men with both bone and soft tissue metastases (55). Lung and liver metastases are associated with a poorer outcome (56), although these sites are less commonly affected.

For patients who undergo RP there are a number of postoperative prognostic factors, apart from GS, that may be recognized by the pathologist. Examples of unfavorable features are perineural invasion, positive surgical margins and EPE including SVI.

1.5.1 EPE

EPE is tumor extension beyond the boundaries of the prostate. EPE is classified as pT3a according to the TNM-system and is a well-established risk factor for recurrence after prostatectomy (57, 58). Efforts have been made to stratify patients with EPE in prognostic subgroups (59, 60). It has been shown that radial extent of EPE predicts prognosis after RP, while perineural invasion at the site of EPE and number of sections or foci of EPE does not (60). Also, non-focal EPE seems to predict a worse prognosis than that of focal EPE (60, 61).

1.5.2 SVI

1.5.2.1 Anatomy of the seminal vesicles and definition of SVI

The seminal vesicles are paired pear-shaped glands, about 5 cm in length. The seminal vesicle consists of a 10-15 cm long coiled tube. The duct of each seminal vesicle joins the vas deferens to form the ejaculatory duct. They are located posterior to the base of the urinary bladder and anterior to the rectum.

Locally advanced prostate cancer may infiltrate the seminal vesicles. The incidence and prognostic effect of SVI varies remarkably depending on the definition of SVI. Previously, tumor cells in the connective tissue surrounding the seminal vesicles was considered sufficient for SVI (62). Today, it is generally accepted that invasion of tumor cells in the muscular coat of the seminal vesicles is required for this diagnosis (Figure 4) (63-65). Further, it has been discussed whether involvement of the intraprostatic portion should be considered SVI. Studies have shown that men with invasion restricted to the intraprostatic portion of the seminal vesicles have a better prognosis than those with extraprostatic SVI and should therefore not be staged as pT3b (66). Accordingly, it was decided at the ISUP consensus conference in 2009 that SVI should be defined as cancer invading the muscular wall of the extraprostatic part of the seminal vesicles (63).

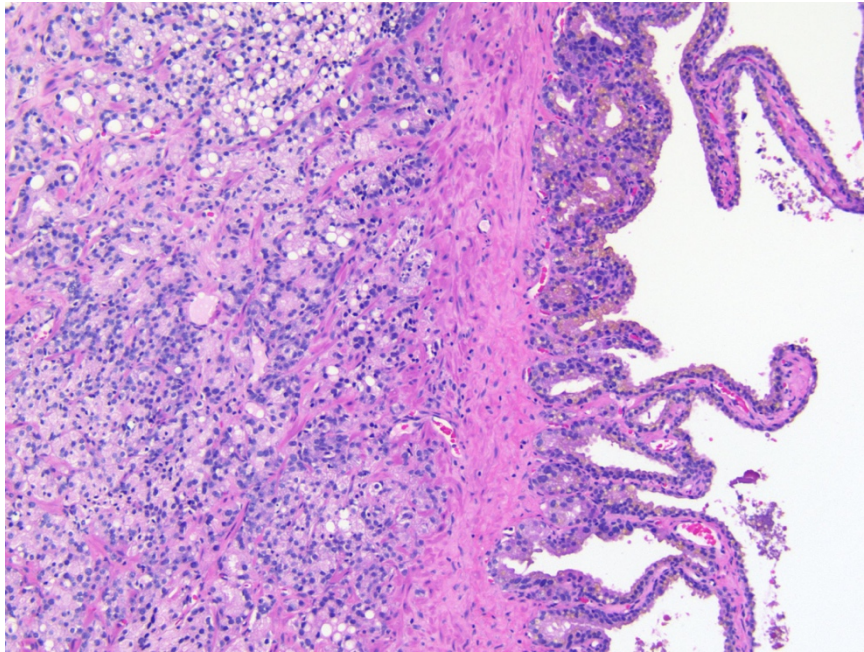


Figure 4. Cancer cells (left) invading the muscular wall of the seminal vesicle. The invasion does not involve the mucosa of the seminal vesicle (right).

1.5.2.2 Route of invasion

Various routes of SVI have been described. Villers *et al* found that the majority of tumors invaded the seminal vesicles via continuous spread along the ejaculatory duct (67). They reported a minority of cases in which tumor first spread outside the prostate and then invaded the seminal vesicles. Only one case in their series had discontinuous isolated foci of tumor in the seminal vesicles. Ohori *et al* further evaluated the mechanisms of SVI and defined three different types of invasion (Figure 5) (68). Type 1 involvement had direct spread along the ejaculatory duct into the inside of the seminal vesicles. Type 2 was defined as invasion through the prostate capsule into the seminal vesicles. This group was further divided into two subgroups; type 2a defined as direct extension of prostate cancer between the base of the prostate and the seminal vesicles and type 2b defined as retrograde growth of prostate cancer along periprostatic nerves. The least common variant, Type 3, had small foci of cancer in the seminal vesicle without continuous growth from the primary tumor. Out of 64 patients with SVI, 17 (27%), 21 (33%) and 8 (13%) were classified as Type 1, 2 and 3 patterns respectively. In 18 patients (28%) it was not possible to distinguish between Type 1 and 2.

In a recent study by Samaratunga *et al* the distribution of cancer growth in the seminal vesicles was examined (69). They evaluated 56 cases of SVI and found that all but one case had invasion to the proximal third of the seminal vesicles. The only case with distal invasion in the absence of proximal invasion had lymphovascular infiltration. The authors suggested

that sampling of the proximal third of the seminal vesicles is sufficient to find practically every case of SVI. However, in cases with lymphovascular invasion and absence of proximal invasion, the entire seminal vesicles should be examined.

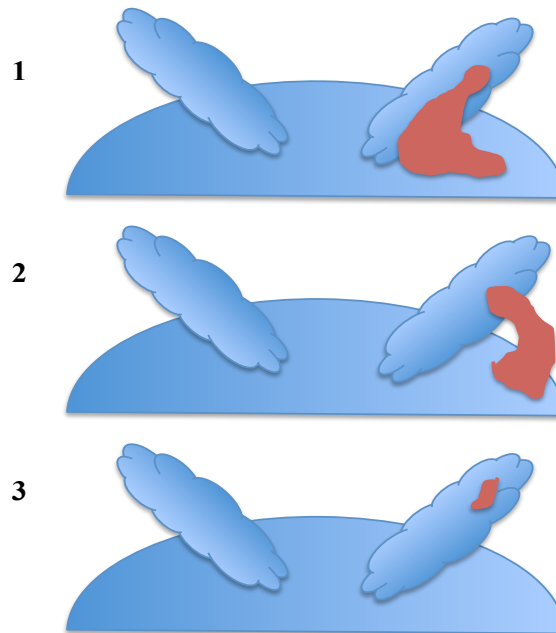


Figure 5. Classification of SVI according to Ohori *et al.* Type 1. Direct infiltration along the ejaculatory duct into the seminal vesicles. Type 2. Invasion through the prostate capsule into the seminal vesicles. Type 3. Discontinuous cancer focus separated from the primary tumor.

1.5.2.3 Incidence and prognostic significance

The incidence of SVI in the literature is highly variable, ranging between 3% and 26% (70-76). It has decreased over the past decades and in recent studies rates between 3% and 7% are commonly reported (69, 71, 77). Traditionally, SVI has been regarded as an indicator of poor clinical outcome and in early studies almost every patient with SVI experienced biochemical recurrence (78, 79). D'Amico *et al* found that SVI was the most significant pathological predictor of s-PSA recurrence with a 2-year s-PSA failure rate as high as 95% after RP (78). Others have reported a 5-year s-PSA progression free rate between 14% and 56% (66, 68, 72, 73, 80, 81). Since the introduction of s-PSA testing patients generally present with more favorable clinicopathological features at time of diagnosis, suggesting improved outcome. Eggener *et al* showed that progression free survival after RP had significantly improved among men with SVI in the s-PSA era. They reported 4- and 7-year biochemical progression free survival rates at 32% and 22% before s-PSA testing compared to 50% and 45% after

(77). However, in another study it was shown that despite decreasing incidence of SVI, the recurrence rate among those tumors remained high with a 5-year biochemical recurrence free survival reported at 36.8% and 36.1% before and during the s-PSA era, respectively (71).

Parameters such as route of invasion, tumor volume, unilateral vs. bilateral involvement of the seminal vesicles, surgical margin status, GS and s-PSA level have been used for stratification of SVI in prognostic subgroups (64, 65, 68, 73, 74, 82). Ohori *et al* examined the prognostic significance of the different routes of invasion (68). When patients with lymph node metastasis were excluded, there was a trend toward better prognosis for patients with Type 3 than with other types; however, the difference was not significant ($p=0.09$). In this group, the tumor size was often smaller and positive surgical margins were less commonly seen. More recently, others have been unable to confirm the prognostic significance of invasion route (64).

Epstein *et al* evaluated 45 cases of SVI and found that tumor volume, extent of SVI or laterality did not correlate with prognosis in this group (65). The only factors that appeared to influence progression were the status of surgical margins and GS dichotomized in GS less than 7 vs. 7 or greater, however, the results were not statistically significant. In a more recent study by the same group, GS, surgical margin status and vascular invasion were significant prognostic factors in multivariate analysis (64). Tefilli *et al* found that positive surgical margins and GS 7 or higher were significant predictors of disease progression analyzing 93 patients with SVI (82). In addition, patients with preoperative s-PSA less than 10 ng/ml had a better clinical outcome. Overall surgical margin status was found to be an independent predictor of disease recurrence in multivariate analysis. However, other studies have shown that positive surgical margins have no effect on progression in men with SVI (73, 80). Salomon *et al* found preoperative s-PSA and the GS of the prostatectomy specimen to be independent prognostic factors, while positive surgical margins did not predict progression (73). There is no evidence that bilateral SVI is associated with worse prognosis compared to unilateral SVI (64, 65).

1.5.2.4 Preoperative diagnosis

Previously, patients diagnosed with SVI preoperatively were usually not considered candidates for surgery with curative intentions due to the high failure rate. In recent years, however, selected patients with advanced disease are more often recommended surgery (83). Recent studies have shown improvements in clinical outcome for patients with adverse risk factors, such as SVI, when treated with RP in combination with postoperative RT (84-86).

For optimized treatment planning accurate prediction of advanced pathology is crucial. Clinical stage, preoperative s-PSA and GS on needle biopsy are often used as predictors of the postoperative pathological stage (52, 87-92). SVI is sometimes detected at preoperative examination by DRE, radiological imaging or targeted biopsies. In addition, biopsy cores positive for cancer at the base of the prostate are associated with higher risk of SVI (93). Yet, how to best diagnose SVI preoperatively is not entirely understood.

Although several studies have shown that seminal vesicle biopsies reliably detect tumor invasion, they are rarely used routinely as SVI is a relatively uncommon finding (94-96). Consequently, the seminal vesicles are rarely targets for biopsies, but sometimes when a biopsy is directed towards the base of the prostate the biopsy accidentally hits the seminal vesicle, which may enable a diagnosis of SVI. Invasion of cancer cells in the muscular wall of the extraprostatic part of the seminal vesicles is required for diagnosis at biopsies (63). Thus, it may be impossible to distinguish between intra- and extraprostatic SVI and ejaculatory duct invasion on needle biopsy. EPE is diagnosed on preoperative needle biopsy when cancer cells are seen infiltrating adipose tissue (97). Apart from these definitive preoperative signs of SVI and EPE, it remains unknown what biopsy characteristics may predict pT3a versus pT3b tumors.

For support in decision making of treatment options, different clinical risk assessment tools are used. Clinical nomograms that include multiple risk variables have been developed for prediction of pathological stage and disease recurrence. Most of them use a combination of the standard predictors of preoperative s-PSA, clinical stage and biopsy GS (52, 87, 92, 98, 99). Other preoperative biopsy findings including percentage cancer in the biopsy cores and percentage of positive cores have been studied, but results are conflicting (89, 91, 100, 101). Bostwick *et al* evaluated length of cancer growth and found that the best preoperative prediction of EPE and SVI was provided by a combination of percentage cancer in the biopsies, s-PSA and GS (89). In multivariate analysis s-PSA and percentage cancer in the biopsy were the most important independent predictors of SVI. Gallina *et al* developed a nomogram for prediction of SVI that included s-PSA, clinical stage, GS and percentage of positive biopsy cores (101). In another study percentage of positive cores was not a predictor of SVI in multivariate analysis (102).

MRI becomes increasingly important in the pre-treatment diagnosing of SVI. Studies have demonstrated that MRI has a high accuracy in predicting SVI and that the predictive value increases when combined with information from nomograms (103, 104). However, MRI alone is not sensitive enough to find all cancers with EPE and SVI. A meta-analysis

evaluating a total of 5677 patients with SVI showed a sensitivity and specificity of 0.58 and 0.96, respectively (105). Also, the experience of the radiologist impacts the accuracy of the SVI diagnosis (104). However, MRI-targeted biopsies outperform standard prostate biopsy in detecting EPE and SVI (106).

1.6 GENOMIC CHANGES IN PROSTATE CANCER

As for all types of cancer, the development of prostate cancer usually involves genomic changes such as point mutations, copy number variations (CNVs) and structural rearrangements. Prostate cancer has a relatively low mutation burden, although advanced disease show a higher mutational burden compared to localized disease (107). Examples of genomic alterations that have been identified in advanced prostate cancer are point mutation or copy number gain of the androgen receptor (*AR*) gene, *TMPRSS2-ERG* fusion, *PTEN* loss, *TP53* mutation, *RB* loss, *MYC* gain and *BRCA2* loss (108). Most of these somatic alterations are overrepresented in metastatic castration resistant prostate cancer (107, 108). The most common structural rearrangement detected in prostate cancer is the gene fusion between the androgen-regulated gene *TMPRSS2* (transmembrane protease serine 2), and a member of the *ETS* transcription factor family (*ERG*, *ETV1* or *ETV4*), which is present in about half of prostate cancers (109-111). This fusion leads to overexpression of the oncogenic *ETS* transcription factor with subsequent development or progression of disease. It has been suggested that *ERG* rearranged cancers are associated with a higher likelihood of disease progression (112), however, in a large prospective cohort study *ERG* overexpression did not predict biochemical recurrence or mortality (113). Further, *ERG* rearrangements have been found in PIN, which suggests that this alteration is an early event and unlikely correlates with aggressive disease (114). Deletion of chromosome 8p, which contains the tumor suppressor *NKX3-1*, and amplification of chromosome 8q, which harbors the *MYC* oncogene, has been reported in cases with localized GS 7 tumors and metastatic disease (115, 116).

PTEN is a tumor suppressor gene that is frequently mutated or deleted in prostate cancer. It is sometimes altered in localized tumors, although it is more commonly found in advanced, castration resistant disease (107, 117, 118). Inactivation of *PTEN* is associated with poor prognosis and therefore early evaluation of *PTEN* status may be used to distinguish patients who need more aggressive treatment (119-122). Alterations in *TP53* are sometimes found in prostate cancer, especially in metastatic disease (107). As for *PTEN* loss, patients with *TP53* mutations have a more adverse clinical outcome. Another important gene is *SPOP*, which is

frequently mutated in prostate cancer (123). *SPOP* mutations lack *ETS* rearrangements, suggesting a distinct molecular class of prostate cancer (123).

Somatic *AR* alterations are detected in metastatic castration-resistant prostate cancer as a consequence of evolutionary treatment pressure and as expected, very rarely in hormone-naïve tumors (108). In the metastatic castration resistant state, prostate tumors remain dependent on AR signaling. By somatic *AR* alterations, the cancer develops resistance to hormonal therapies (124). New treatments that target the AR (enzalutamid) or production of testosterone (abirateron) have been developed and successfully introduced for patients with metastatic castration-resistant disease. However, sooner or later the tumor becomes resistant to these treatments through additional alterations in *AR* or by other mechanisms, for example through neuroendocrine differentiation (125).

Since the introduction of next generation sequencing the understanding of the molecular basis of prostate cancer has rapidly increased. Many advances have been made in the understanding of tumor progression on a molecular level. This enables subclassification of the tumors and will be important for personalized medicine. For example, patients with intermediate-risk disease and molecular changes indicating poor prognosis may be suited for curative treatment instead of active surveillance.

Genetic susceptibility for developing prostate cancer is further presented in *Risk factors*.

1.7 HETEROGENEITY AND ORIGIN OF MULTIFOCAL DISEASE

Prostate cancer is usually a multifocal disease with more than one intraglandular tumor focus in 60-90% of cases (36, 126). It has been widely discussed whether separate tumor foci have different clonal origins with separate somatic profiles, or if the tumors have a monoclonal origin, meaning that a specific clone spread and result in distinct but genetically similar tumors. In order to investigate the somatic relations of separate tumor foci, molecular characteristics have been mapped by several research groups; majority of them indicating clonal independency among separate tumor foci within the same prostate gland (37, 127, 128). Early studies in this field used methods such as analysis of polymorphic microsatellite regions and fluorescent in situ hybridization (FISH) targeting centromeric regions for selected chromosomes.

Cheng *et al* examined the pattern of allelic loss in patients with separate tumor foci using four microsatellite polymorphic markers (three for a putative tumor suppressor gene and one for

the *BRCA1* gene) (37). In 15 out of 18 patients a random pattern of allelic deletion was observed in spatially separated tumor foci. In another early study, FISH was used to study numerical chromosomal anomalies in multiple foci of prostatic carcinoma in 40 RP specimens (129). A widespread genomic heterogeneity with different chromosomal abnormalities was seen within the same prostate gland. More recently, Kobayashi *et al* sampled tumor foci of prostatectomy specimens with laser capture microdissection (LCM) and used comparative genomic hybridization for CNV analysis (127). They found that separate tumor foci had different CNVs that sometimes overlapped but never were entirely identical. In line with previous studies, they concluded that multifocal prostate cancer is a polyclonal disease.

Analyses of the prostate cancer specific *TMPRSS2-ERG* gene fusion have been used in heterogeneity studies indicating a high level of genomic heterogeneity. Mehra *et al* used FISH to analyze *TMPRSS2* rearrangement in a total of 93 tumor foci from 43 RP specimens (130). Of the 43 cases, 30 (70%) showed *TMPRSS2* rearrangement within at least one tumor focus. Furthermore, 70% of the rearranged cases showed discrepancy in rearrangement pattern in separate tumor foci indicating clonal heterogeneity.

Although the majority of these studies indicated clonal independence, the methods are not sensitive enough to fully understand the somatic relationship between tumor foci. A single structural alteration or chromosomal abnormality represents only one event and does not provide information on remaining parts of the genome. More recently, different groups have used next-generation sequencing in attempts to better understand the molecular background of multifocal disease. In genome-wide studies the analysis is not restricted to single events, but changes across the entire genome are analyzed. This provides an opportunity to a more detailed evaluation of the heterogeneity pattern.

Lindberg *et al* performed whole-exome sequencing on multiple tumor foci in four individuals (128). The tumor foci did usually not share any of the identified SNVs and their results indicate a high level of heterogeneity and somatic independency between foci. In another recent study, 89 tumor foci in 41 men were examined and in 76% of cases none of the foci shared any point mutations (131). In addition, the few shared mutations were rarely in genes that are critical for the development of cancer.

Although the majority of studies have hinted a multiclonal origin, a few have claimed the opposite. Boyd *et al* performed array-based CNV analysis on 18 cases of clinically localized prostate cancer and found identical copy number changes shared in all foci within the same

case (132). They suggested that although genomic changes accumulate independently in separate tumor foci as cancer progresses, it is unlikely that all foci have the exact same genomic changes even if they origin from the same initial clone. Prostate carcinogenesis has also been suggested to be a result from a field effect with an abnormal mutational process from which prostate cancer develops and clones further branch into subclones (133).

In a study by Boutros *et al*, 74 patients with index tumor of GS 7 were examined (115). Copy number analysis revealed extensively heterogeneous profiles between patients. Five cases were further sampled and subjected to whole-genome sequencing of 2-9 spatially separated tumor foci. A pronounced intertumoral heterogeneity was found when analyzing structural alteration, CNVs and SNVs. No shared CNVs and very few shared SNVs were observed between tumor foci. They further tested a few prognostic markers for intermediate-risk prostate cancer to evaluate the concordance between foci. For example, loss of *NKX3-1* was reported in two of five foci in one of the cases. This indicates that information of genomic-based prognostic markers from a single focus should be interpreted with caution. They concluded that the high degree of intratumoral heterogeneity with difference in driver alterations between foci implies a multiclonal origin of prostate cancer.

The majority of studies that used high-resolution sequencing methods have shown that the somatic heterogeneity between foci is high and that genomic alterations rarely are shared between foci. Therefore, multifocal prostate cancer is nowadays commonly regarded as a multiclonal disease. With new methods it has also been demonstrated that the heterogeneity is high within a single tumor focus. In prostate cancer there is often a dominant, high-grade index focus that is relied on in prognostication. Index foci have recently been reported to be somatically heterogeneous, with many alterations present in subclonal populations (134).

1.8 THE CLONALITY OF METASTATIC PROSTATE CANCER

Studies on the genomic heterogeneity of prostate cancer and the clonal relationship in metastatic disease are important in order to understand the pathogenesis of the disease. In addition, it has major implications in the clinical setting as targeted treatments on driver genomic changes become increasingly important. In contrast to multifocal localized disease, which seems to have a multiclonal origin with separate tumor foci arising independently, studies on metastatic disease have indicated a monoclonal origin (107, 116, 135, 136). Similar to studies on multifocal disease, this question was previously studied with

chromosomal analysis using FISH and CNV analysis by comparative genomic hybridization and it was early suggested that a single tumor clone is responsible for progression (129, 135).

With next-generation sequencing the opportunity to study the clonal relationship between primary tumor and metastasis has improved significantly. These high-throughput methods may also provide a time-line for somatic events, which is valuable when studying clonality. Haffner *et al* used whole-genome sequencing and followed one case of lethal prostate cancer through longitudinal sampling from the primary tumor and its metastases (137). They were able to identify genomic characteristics of the metastatic cell clone and traced the origin back to a focal area in the primary cancer. Surprisingly, a small focus with a Gleason grade 3 cancer showed the same genomic alterations as the metastases, such as mutations in *PTEN*, *TP53* and *SPOP*.

Although the majority of studies have indicated monoclonal seeding of metastases, Gundem *et al* showed by detailed sampling of patients with castration-resistant metastatic disease that multiple clones are involved in metastatic seeding (138). In three of ten cases they found that more than one subclone from the primary tumor achieved metastatic potential. In this study metastasis-to-metastasis was common and occurred by two mechanisms; either through de novo monoclonal seeding or through the transfer of multiple clones between metastatic sites.

The somatic concordance between spatially separate metastases in patients with castration-resistant metastatic prostate cancer was recently shown to be high (116). In this study genomic alterations were recurrent in *AR*, *ERG*, *TP53*, *RBI*, *SPOP*, *CHD1* and *ZBTB16*. These alterations have previously been reported by others in metastatic disease (107). In addition, gain in regions of chromosome 8q, including *MYC*, and loss in regions of 8p was frequently seen. All mutations were not found in every single metastatic deposit, however, driver mutations and potential molecular targets were usually present in every metastasis. In line with this study, it has been demonstrated that DNA methylations among men with lethal metastatic prostate cancer are maintained in all metastases of the same individual (139). These studies indicate that tumor sampling from a single metastasis may be representative of the important genomic alterations of the cancer.

1.9 TREATMENT

1.9.1 Curative treatment

Localized disease is classified according to different risk groups (see *Prognosis*) and treatment recommendations are based on the risk category in combination with health state and age of the patient. It can be cured either by RP or RT. Both treatments are restricted by side effect such as urinary incontinence and erectile dysfunction. Active surveillance is an option for patients with low risk disease. Patients with expected survival under 10 years and low or intermediate-risk disease may be recommended watchful waiting, which means that hormone treatment is initiated when needed without curative intention. A large randomized study showed no difference in prostate cancer specific mortality after ten years for men with localized disease treated with active surveillance, RT or RP (140).

1.9.1.1 Surgery

RP is either done by open retropubic, laparoscopic or robot-assisted laparoscopic approach. No significant differences in oncologic or functional outcomes have been shown (141, 142). Nerve-sparing technique is used when indicated by features of the tumor, age of the patient and preoperative erectile function. If there is a high risk of EPE, nerve-sparing procedure is not recommended. The role of pelvic lymph node dissection during RP remains controversial (143).

1.9.1.2 RT

Curative external RT is given to patients with intermediate- or high-risk localized prostate cancer. The radiation techniques have rapidly improved in recent years. Nowadays, RT is more precise and higher doses can be given with lower risk of side effects. Patients with prostate cancer usually receive up to 78 Gy in 2 Gy fractions, although hypofractionated doses have shown similar effect for patients with intermediate-risk tumors (144). HDR (high dose rate) brachytherapy is sometimes combined with external radiation. For high-risk patients with T3 tumors, RT in combination with hormonal therapy is usually recommended over surgery (145). Neoadjuvant and adjuvant hormone treatment is given to the majority of patients with high-risk tumors and a few with intermediate-risk tumors.

1.9.1.3 Adjuvant RT after RP

Adjuvant RT after RP is given to patients with s-PSA <0.1 ng/ml but high risk for postoperative recurrence. The length of positive surgical margin is a prognostic factor for

biochemical recurrence after RP (146). In Sweden, adjuvant RT is sometimes considered for patients with extensively positive surgical margins (>3mm) without lymph node metastases.

1.9.1.4 Salvage RT

For patients with slowly rising s-PSA (two consecutive values over 0.2 ng/ml) after RP and suspected local recurrence, salvage RT is commonly recommended. Although the majority of patients with local recurrence have positive surgical margins, local recurrence may also occur in patients with negative margins. Salvage RT is given with curative intention. If the patient has a high GS or s-PSA hormone therapy may be added.

1.9.1.5 Hormone treatment

Androgen deprivation therapy (ADT) is used both in curative and palliative treatment. Castration is achieved either surgically by orchidectomy or medically through luteinizing hormone releasing hormone (LHRH) agonist/antagonist. Antiandrogens (bicalutamide) are commonly used in combination with castration as neoadjuvant and concomitant treatment during curative RT.

1.9.2 Palliative treatment

The vast majority of patients with metastatic prostate cancer initially respond to castration therapy, which is always used as a treatment base in the palliative setting. Recent studies have shown a survival benefit for patients with metastatic hormone sensitive prostate cancer who receive docetaxel or abirateron (147, 148). However, sooner or later the disease progresses to a castration-resistant state (149). Mechanisms of resistance include *AR* overexpression, *AR* mutations and increased intratumoral steroidogenesis (150). Today, there are a number of different treatment options for patients with metastatic castration-resistant disease, including chemotherapy (docetaxel, cabazitaxel), second-generation antiandrogens (enzalutamide, abirateron) and radioisotope (radium-223).

1.10 SUMMARY

There is a need of additional biomarkers for treatment stratification of prostate cancer patients. Increased knowledge of histological features and genomic changes associated with aggressive disease would be helpful for treatment planning. With the stage shift that has occurred over the last decades, the incidence of locally advanced prostate cancer has decreased. SVI has traditionally been regarded as an ominous prognostic indicator. However, the group of cancers with SVI is heterogeneous and not all patients with SVI experience recurrence of disease. Further stratification is needed in order to better predict prognosis and optimize treatment for this patient group.

The morphological and genomic profile of clones with metastasizing potential has not been fully identified. More studies are needed to describe the characteristics of these clones.

Prostate cancer is a somatically heterogeneous disease. Multifocal tumors often present different molecular profiles in spatially separated tumor foci. Clinical use of predictive and prognostic genomic biomarkers becomes increasingly important to optimize and personalize treatment. Core biopsy assessment in the initial diagnostic setting is an important step in the treatment planning process. In order to rely on the molecular biomarkers from the biopsies it is important that there is a high concordance between genomic findings in biopsies and prostatectomy specimens. However, it is still unclear how well the preoperative biopsies represent the somatically heterogeneous tumor foci in the prostate.

2 AIMS

The overall aim of this thesis was to investigate prognostic factors and genomic heterogeneity of pathological features in locally advanced and multifocal prostate cancer. It was further to analyze the utility of preoperative biopsies for prediction of somatic aberrations in multifocal prostate cancer. The specific aims were:

1. To evaluate the correlation between histopathological variables and the risk of biochemical recurrence after RP in men with SVI.
2. To study the genomic heterogeneity of prostate cancer and its relation to the clonality of metastatic disease.
3. To study the clinical outcome and analyze the preoperative needle biopsy findings in patients with SVI compared to patients with EPE alone.
4. To evaluate the genomic heterogeneity in multifocal prostate cancer and to analyze how well the preoperative biopsies represent the somatically different foci found in the prostatectomy specimen.

3 MATERIAL AND METHODS

3.1 TISSUE COLLECTION AND PREPARATION (STUDY I, II, IV)

All RP specimens included in Study I, II and IV underwent the same preparation process. The specimens were formalin-fixed overnight, inked and totally embedded. Sections were cut horizontally at 4 mm thickness and either whole-mounted or cut into 2-6 segments. The specimens were then dehydrated and paraffin embedded. Sections were cut at 4 μ m and stained with hematoxylin and eosin (H&E). Cancer was outlined on the slides with black Indian ink.

3.1.1 Study I

All patients included in Study I underwent RP at the Karolinska University Hospital between May 1998 and December 2005. Patients were excluded if they had received neoadjuvant treatment (hormonal or RT) or undergone transurethral resection prior to surgery. Cases with unavailable histological slides or clinical follow-up were also excluded. After exclusion 1050 cases remained for analysis, including 60 cases with SVI. Due to changed operation technique during the inclusion period the seminal vesicles were resected completely in 19 cases and partially in 41 cases. Partial resection was done to shorten operation time and reduce the risk of nerve damage.

3.1.1.1 Protocol and definitions

Every case with SVI was reviewed according to a prepared protocol. The following features were recorded: laterality, route of invasion, distribution in the seminal vesicle wall, extent (intraprostatic vs. extraprostatic), diameters of the seminal vesicle tumor deposits, tumor area, positive surgical margins in the seminal vesicle, invasion of vas deferens and GS.

Measurements and definitions of the features are described in detail here:

- *SVI* was defined as tumor invasion of the muscle wall of the extraprostatic part of the seminal vesicles. When prostatic tissue and seminal vesicle tissue was seen on the same slide, it was considered to be *intraprostatic* seminal vesicle tissue.
- *Laterality* was recorded as uni- or bilateral invasion.
- *Route of invasion* was evaluated according to the definitions used by Ohori *et al* (68). Type 1 is defined as growth along the ejaculatory ducts into the seminal vesicles. In Type 2 there is tumor extension at the base of the prostate into the connective tissue

surrounding the seminal vesicle. Type 3 is discontinuous metastases to the seminal vesicle.

- *Level of tumor invasion* of the seminal vesicle was registered (Figure 6). Tumor was present in the muscular wall alone or in combination with invasion of the mucosa and/or the connective tissue surrounding the seminal vesicle. Mucosal invasion was defined as cancer infiltrating between tubular seminal vesicle glands.
- *The diameters* of the tumor area in the seminal vesicle were measured. The first diameter was measured from the base to the tip of the seminal vesicle and the second perpendicularly to the first.
- *The tumor area* was determined using grid paper. The tumor contours were copied onto grid paper with one dot per millimeter and all dots within the tumor area were counted. The number was used as a surrogate marker for the tumor area.
- *Status of the surgical margins* of the seminal vesicles was recorded.
- *Invasion of vas deferens* was noted. In some cases the vas deferens could not be identified and presence of invasion was considered indeterminable.
- *GS* was registered both for the main tumor and for the seminal vesicle component.

Follow-up data was obtained from patient records at the Karolinska University Hospital. Biochemical recurrence was defined as two consecutive s-PSA values ≥ 0.2 ng/ml. S-PSA was measured according to a standardized protocol 6-8 weeks after RP and once or twice annually thereafter.

3.1.1.2 Histological evaluation

All seminal vesicle slides were reviewed first by the author and later together with a senior pathologist (Lars Egevad, LE).

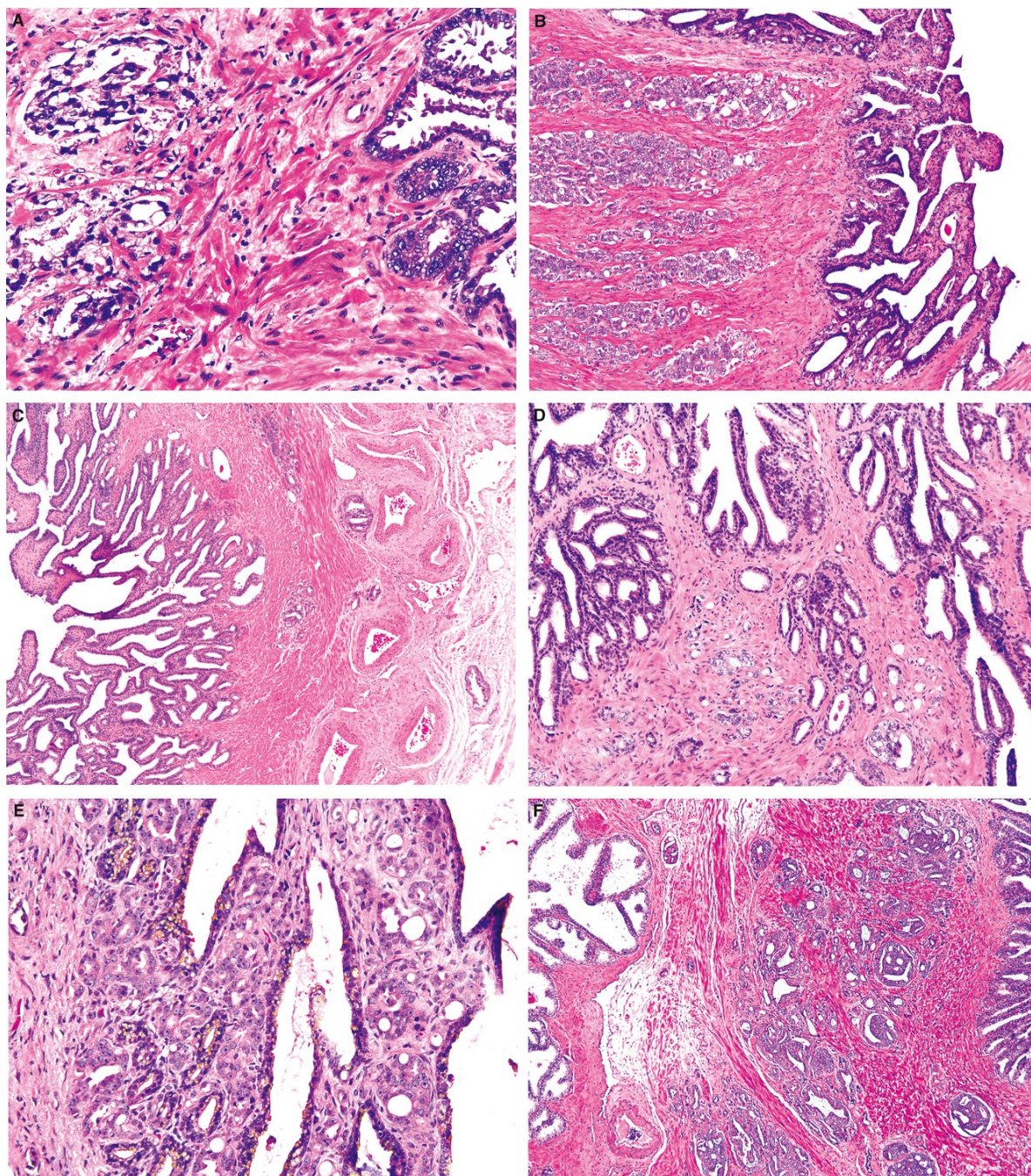


Figure 6. H&E sections of different types of seminal vesicle invasion. (A) Cancer glands (left) invading the muscular wall of the seminal vesicle. (B) Muscular wall invasion of cancer cells (left). The seminal vesicle mucosa (right) has not been invaded. (C) Prostate cancer glands invading the muscular wall and the connective tissue surrounding the seminal vesicle (right). (D) Cancer invasion of the mucosa of the seminal vesicle. Cancer glands invade between seminal vesicle glands. (E) Mucosal invasion. (F) Invasion of the intraprostatic part of the seminal vesicle. Seminal vesicle (right) and prostate glands (left).

3.1.2 Study II

3.1.2.1 Previous study

In a previously published study, our group analyzed autosomal and mitochondrial DNA of prostate cancers (151). Part of the aim was to validate the clonal relationship between primary

tumor and metastases in two cases (one aggressive neuroendocrine tumor with paired bone metastasis and one adenocarcinoma with two lymph node metastases) by cellular frequency estimation of all SNVs found in those cases. The neuroendocrine tumor was found to have a strong monoclonal relationship between the primary tissue and metastasis, with high degree of shared mutations. However, in the second patient it was not possible to find a common somatic denominator between the fresh-frozen primary tumor tissue obtained after RP (SWE-54A) and fresh-frozen right/left pelvic lymph-node metastases (SWE-54B/C). Therefore, this case became the subject of a more thorough investigation in order to find the specific clone responsible for seeding the metastases. The patient and the tumor characteristics are described in detail below.

3.1.2.2 Patient, histology and tissue preparation

The patient analyzed in Study II underwent RP and pelvic lymphadenectomy at the Karolinska University Hospital, Stockholm, in 2011. The preoperative s-PSA was 16 ng/ml and biopsies showed an acinar adenocarcinoma of the prostate, GS 4+5=9 with a total cancer extent of 6 mm cancer. Postoperative histological examination revealed a bilateral adenocarcinoma with GS 4+3=7 and tertiary Gleason pattern 5. The lymphadenectomy specimen was received unfixed and fresh tissue was harvested and frozen. Bilateral lymph node metastases were found in the histological sections. The main tumor in the RP specimen originated from the PZ although a small separate tumor focus was also found in the TZ. The postoperative stage was pT3bN1. Forty-five morphologically distinct foci were identified for the genomic analysis (Table 2). The spatial localizations of the separate tumor foci are seen in Figure 7.

Table 2. Tissue area harvested with LCM

Areas	Histopathological feature
1–29	Main tumor
7–9	Intraductal carcinoma
30–34	Separate foci in peripheral and transitional zone
10, 18	Extraprostatic extension
27–29	Seminal vesicle invasion
35–39	High grade PIN
40–42	Atrophy
43–44	Benign prostatic hyperplasia
45	Basal cell hyperplasia (with spots of squamous cell metaplasia)
46	Normal tissue, epithelial cells
47	Normal tissue, stromal cells

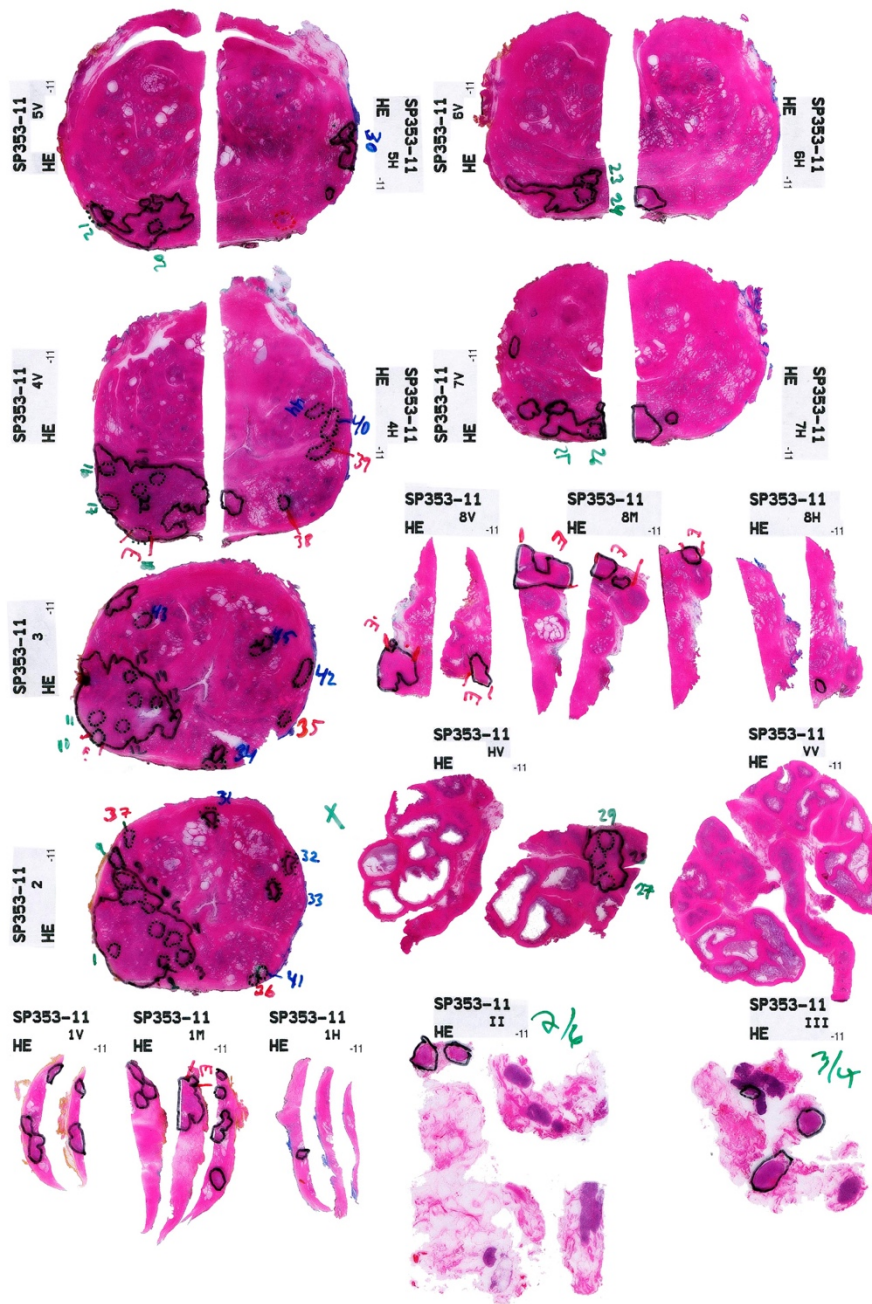


Figure 7. Whole-mount sections of the prostatectomy specimen examined in Study II. Cancer is outlined with black Indian ink. Areas collected with LCM are marked and numbered.

The paraffin blocks were serially sectioned at 10- μ m and mounted on UV-treated membrane slides. The slides were stained with H&E and stored at 4°C before LCM.

3.1.3 Study IV

Eleven patients with multifocal disease who had undergone systematic biopsies followed by RP at the Karolinska University Hospital between 2011 and 2012 were included in Study IV.

At least two distinct major tumor foci were observed in every case. In each case, one to three biopsy cores with the greatest tumor extent were selected for analysis. Only cores with more than 2 mm cancer were analyzed. When possible, cores from both the left and the right side of the prostate were included.

Fresh frozen tissue was harvested from the RP specimens by cutting a horizontal section through the prostate gland using a double-bladed knife. The tissue slice was split into smaller blocks, embedded in Optimal Cutting Temperature (OCT) compound, snap-frozen in liquid nitrogen and stored at -80°C . Fresh frozen tissue was sectioned in 5 and 10 μm serial sections and a total of 100 μm thickness of the tissue block was collected from every focus. The tumor foci were macroscopically dissected and sections collected in a tube. The sample was stored at -80°C before extraction. The formalin-fixed paraffin-embedded (FFPE) tissue was prepared as described above. Both frozen and FFPE sections were stained by H&E. The paraffin blocks of the biopsies and the prostate were cut in serial sections at 10 μm and the tumor foci were macroscopically dissected. In total, four sections were collected in a tube and stored at 4°C before extraction. Control sections of all tissues were examined microscopically to verify a $\geq 25\%$ tumor content.

The FFPE samples from biopsy and RP specimens were designated BX and RP FFPE, respectively, and the frozen samples were labeled RP frozen.

3.2 STUDY POPULATION AND DATA COLLECTION (STUDY III)

The third study of this thesis was a registry study based on data retrieved from the NPCR, which covers 98% of all prostate cancers that are diagnosed in Sweden. Data on clinical and pathological features as well as primary treatment is registered in NPCR. In addition, information on the preoperative needle biopsies has been recorded systematically since 2009. In Study III, data were obtained on all men who were diagnosed with prostate cancer between 2000 and 2012 and who underwent RP as primary treatment. Patients with stage pT3a and pT3b were analyzed and compared. Furthermore, the preoperative needle biopsies of patients with pT3a and pT3b who were diagnosed from 2009 to 2012 were analyzed.

3.2.1 Outcome measurements

In order to analyze outcome, NPCR data were merged with information from the Swedish Cause of Death Registry and Prescribed Drug Registry. Clinical progression was defined as initiation of postoperative treatment with RT or ADT. Patients who received treatment within

two years after RP were excluded since they possibly were treated with adjuvant intention. Patients who received ADT were identified through the Prescribed Drug Registry, which registers all prescribed medications retrieved by patients. This register was started in 2005 and left truncation was therefore needed on this data. Radiation doses less than 60 Gy were considered to be palliative treatment. Data on death from all causes and death from prostate cancer were retrieved from the Swedish Cause of Death Registry and also used as endpoints.

3.3 LCM (STUDY II)

One of the challenges of preparing tumor tissue is to separate and collect the specific cells of interest from the surrounding tissue. This issue is particularly pronounced in prostate carcinoma since the tissue usually is very heterogeneous and tumor glands often infiltrate between normal glands. Macrodissection may lead to a low tumor purity, which aggravates analysis of data. LCM allows for isolation of specific cells or glands from tissue under direct microscopic visualization. Through this method it is possible to ensure a high tumor purity of the specific cells of interest. LCM was developed at the National Cancer Institute of the National Institutes of Health in Bethesda, USA in the late 90s (152). A thermoplastic membrane is placed over a tissue section, on which the cells of interest adhere using a pulse from an infrared laser. This film is then directly transferred to for example a buffer for downstream analysis.

In Study II a LCM instrument from Zeiss was used for microdissection. The tissue sample is cut and catapulted from the slide to a microfuge tube cap that collects the tissue. The slides were examined under the microscope and the glands of interest marked and dissected into the adhesive capsule (Figure 8). During microdissection the original slides were continuously examined under a microscope to ensure that the right glands were harvested. In a few cases the area was examined, marked and cut in the LCM microscope but instead of being catapulted into the adhesive capsule, it was transferred to the capsule using a sharp knife. This method was used to yield larger amounts of DNA in cases where the tumor cells were tightly packed to ensure a high tumor purity of the samples. All tumor samples were estimated to contain 80-100% tumor cells. Samples were collected according to Table 2. Six foci were excluded because the morphology could not be confirmed after re-cutting of the blocks. Photographs were taken before and after dissection. Areas that showed a slightly changed morphology compared with the original slides were always discussed with a senior

pathologist (LE). The dissected samples were kept in a box protected from light at room temperature (18–22°C) until they were prepared for DNA extraction.

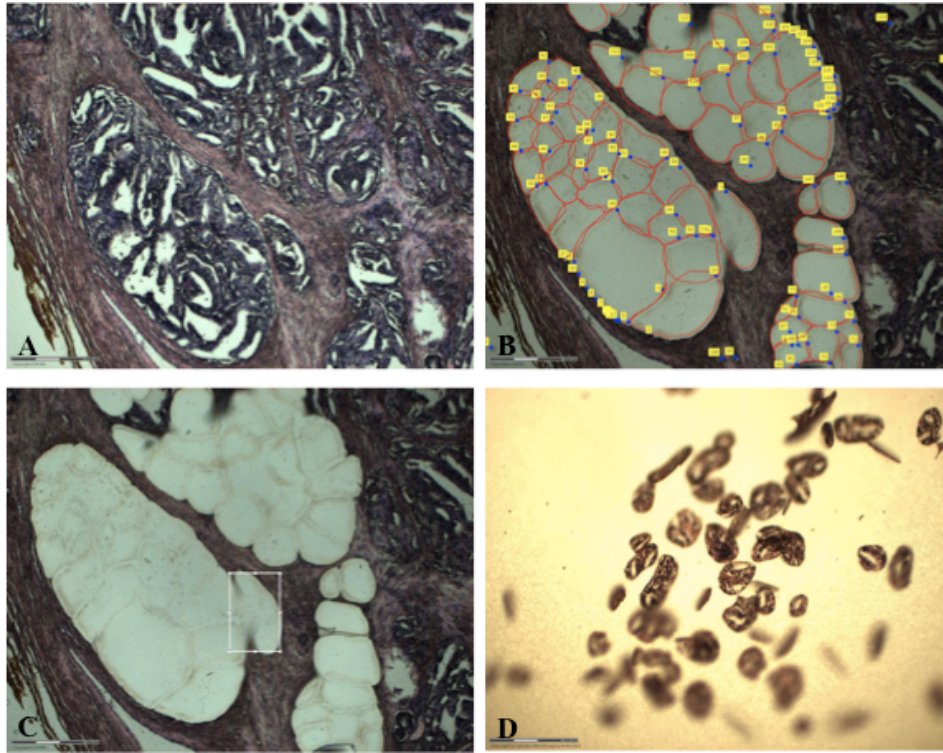


Figure 8. Pictures from the LCM process. (A) Glands subjected for LCM before cutting. (B) Small areas with tumor cells are drawn and cut with the laser. (C) The pieces with tumor cells have now been cut out and catapulted into an adhesive capsule. (D) Tumor samples collected in the adhesive capsule.

3.4 DNA EXTRACTIONS (STUDY II AND IV)

3.4.1 Study II

DNA was extracted using QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's instructions. After extraction the samples were eluted in RNase-free water and stored at -18°C before library preparation and sequencing. DNA concentrations were measured using a Qubit 2.0 fluorometer. The DNA amounts of the samples ranged between 1.3 to 51.6 ng, with highest amounts in the macrodissected areas. Six samples were lost due to manufacturing flaws (leakage from the adhesive capsules during extraction).

3.4.2 Study IV

In Study IV a total of 69 tumor samples were extracted for DNA. The samples consisted of 22 FFPE biopsies, 25 FFPE tumor foci and 22 fresh frozen tumor foci. DNA extraction was performed using the Allprep FFPE kit (Qiagen) according to the manufacturer's protocol.

3.5 LIBRARY CONSTRUCTION, SEQUENCING AND BIOINFORMATICS (STUDY II AND IV)

3.5.1 Study II

The sequencing libraries for the fresh-frozen primary tumor tissue (SWE-54A) and the right/left lymph-node metastases (SWE-54B/SWE-54C) were previously prepared and processed (151). The construction of libraries for microdissected tissues was done using a ThruPLEX kit (Rubicon Genomics, USA) according to a standard protocol.

Low-pass whole-genome sequencing was done to detect break-point regions (BPRs). A BPR is a region where the sample sequence alters from the reference sequence and marks a start of an amplification or a deletion event. A total of 385 BPRs were identified, which were used for the construction of a somatic phylogenetic tree. Nine tumor samples contained fewer than five BPRs and were excluded. In addition, eleven foci were lost during tissue harvesting, library preparation or quality control of the data. In total, 28 samples, including SWE-A/B/C, were further analyzed. The somatic relationship between every tumor foci and the metastases was presented in the phylogenetic tree. The right and left lymph node showed a high degree of similarity with 80% (62 events) of the BPRs shared between samples.

3.5.2 Study IV

The library preparation and sequencing steps were done in collaboration with SciLifeLab, Solna, as previously described (153). Briefly, DNA was used to create sequencing libraries using ThruPLEX kit (Rubicon Genomics, USA) and DNA profiling was performed with in-solution hybridization capture and targeted sequencing (SeqCap EZ system, Roche Nimblegen, USA). Targeted sequencing of 289 genes was done. Downstream bioinformatics, including basic quality control and identification of mutations, CNVs and structural variations, were also performed as described (153). Mutations and structural variations were manually annotated for each case for the identification of putative driver alterations.

Tumor samples that shared a CNV pattern or at least one driver alteration with allele fraction $\geq 0.25 \times$ tumor purity were considered to have a common somatic denominator, i.e. they were

classified as the same cancer. In a few samples it was hard to draw conclusions on somatic denominator due to insufficient amount of DNA, low coverage ($<100\times$) and/or low tumor purity ($<20\%$). Variants in samples with tumor purity <0.2 were considered clonal with an allele fraction ≥ 0.05 . Tissues that did not harbor any clonal variant, but shared non-clonal events with another cancer, were designated as a possible match.

3.6 IMMUNOHISTOCHEMISTRY (STUDY II)

Immunohistochemistry is a method for detection of proteins in tissue sections. The method was used in Study II, which together with morphological evaluation confirmed the diagnosis of IDC-P. The basic principle of immunohistochemistry involves antibodies binding specifically to antigens in the tissue. This binding can be visualized in several ways. Most commonly, the antibody is conjugated to an enzyme, which allows for visual detection when activated.

One of the major criteria for the diagnosis of IDC-P is entire or partial preservation of the basal cell layer. Therefore, immunohistochemical staining was performed for a typical basal cell marker, p63, and alpha-methylacyl-CoA-racemase (AMACR), commonly strongly expressed in prostate cancer. A positive staining for p63 in a basal cell distribution combined with a positive staining for AMACR in luminal cells would be suggestive of the diagnosis IDC-P. The staining was done according to standard protocols.

3.7 STATISTICAL ANALYSES (STUDY I AND III)

Statistical analyses of Study I and III were performed using the program R statistics (The R Foundation for Statistical Computing, Vienna, Austria). Cox regression models were used for time-to-event analyses of biochemical recurrence in Study I and death from prostate cancer, death from any cause or clinical progression in Study III. Wilcoxon's signed-rank test was used for paired comparisons in Study I. Hazard ratios were calculated and Kaplan-Meier curves were created to compare progression-free survival between patients with and without SVI and mucosa invasion. In Study III missing values from the registry data were imputed using multiple imputation based on chained equations (154). Five imputation datasets were created. pT stage was imputed for true missing values and in cases with no distinction between pT3a and pT3b. The means of the imputation datasets were used for generating Kaplan-Meier curves to compare patients with tumor stage pT3a and pT3b. In order to

evaluate any change in clinical outcome over time, the progression-free survival of patients with pT3a and pT3b tumors was analyzed for each year from 2000 to 2012. Mann–Whitney U test and chi square test were used to compare biopsy data between the different stage groups. In both studies, p-values of less than 0.05 were considered statistically significant.

3.8 ETHICAL CONSIDERATIONS

Study I-IV were approved by the Regional Ethic Review Board in Stockholm (2006/1014-31, 2010/710-31/2, 2013/153-31, 2015/0147-32).

4 RESULTS AND DISCUSSION

4.1 STUDY I: CANCER INVASION TO THE SEMINAL VESICLE MUCOSA IS ASSOCIATED WITH A HIGH RISK OF BIOCHEMICAL RECURRENCE

4.1.1 Main findings and general discussion

In the first study we reviewed a consecutive series of RP specimens from the Karolinska University Hospital to identify cases with SVI. Histopathological features of SVI were analyzed in order to find possible prognostic factors. Of the 1050 RP cases reviewed, 60 (5.7%) had SVI, which is in line with other contemporary studies, reporting incidences of SVI in around 3-9% of RP specimens (71, 74). The incidence of SVI has decreased during the last decades as a result of earlier detection of cancer since the introduction of s-PSA testing (71). A current trend towards surgery in patients with more advanced stages may, however, again increase the proportions of patients with stage pT3.

SVI was associated with a poor prognosis (HR 1.7, 95% CI 1.1-2.6, $p=0.015$), with biochemical recurrence in 38.3% of cases. The slope of the Kaplan-Meier curve was relatively steep the first 20 months for patients with SVI, but then leveled out and became more or less parallel with the curve of patients without SVI (Figure 9). This indicates there is an increased risk of early recurrences in pT3b disease, although the reason for this has not further been evaluated. It would be of interest to study characteristics of the group of men with early recurrences.

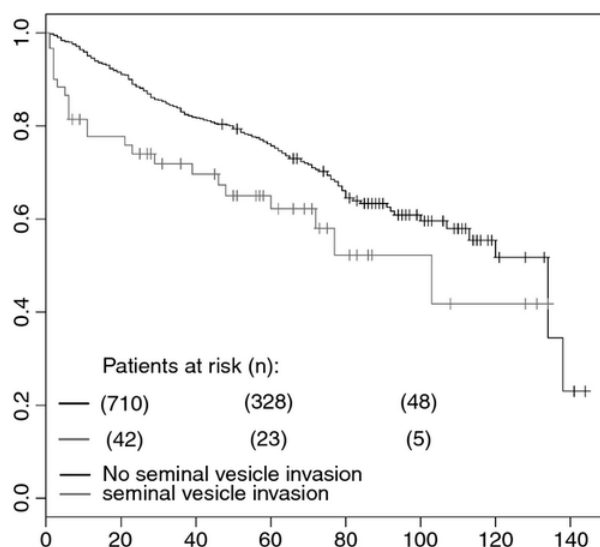


Figure 9. Kaplan-Meier curve estimating the recurrence-free survival of patients with and without seminal vesicle invasion. HR 1.7 (95% CI 1.1–2.6, $p = 0.015$). In total, 60 patients with seminal vesicle invasion and 990 patients without seminal vesicle invasion were analyzed.

SVI of prostate cancer is a well-established risk factor for biochemical recurrence after RP, which was confirmed in this study. However, the mechanism behind its prognostic significance remains unknown. One possibility is that the seminal vesicle is a favored site for further tumor cell dissemination. Potter *et al* suggested that the dismal prognosis was related to occult lymph node metastases missed in the routine microscopic examination, but they were not able to confirm their hypothesis by immunohistochemical and molecular methods (155). Another proposal is that the tumor cells require certain characteristics in order to invade the seminal vesicles, such as the ability to infiltrate another tissue type. In that case, invasion of the seminal vesicles may be considered a surrogate marker for tumors with aggressive potential. In our study the seminal vesicle component always contained the highest Gleason pattern present in the main tumor, and a GS lower than 7 was never seen in the seminal vesicle component (Figure 10). It has been suggested that the poorer clinical outcome in patients with SVI depends on a higher GS. However, SVI usually remains a significant risk factor in multivariate analyses when adjusting for GS (64).

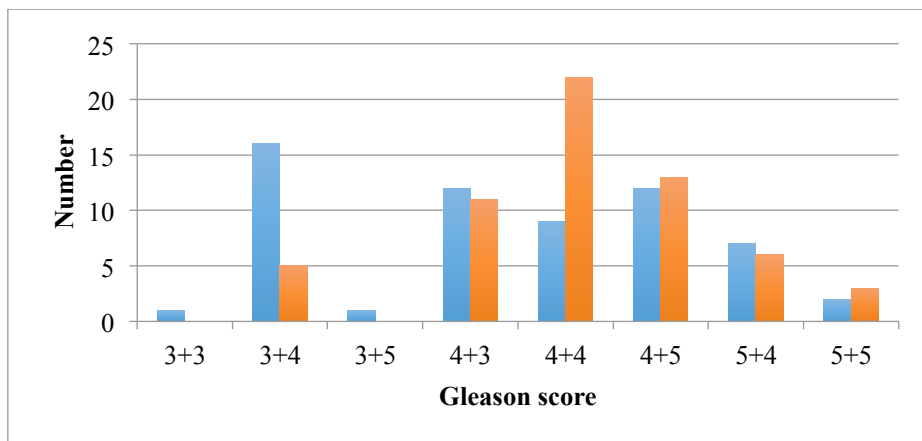


Figure 10. Distribution of GS in the main tumor and the SVI component. Blue staple: Main tumor. Red staple: SVI component. In the majority of cases the SVI component had the same GS as the main tumor. A GS lower than 7 was never seen in the seminal vesicles.

Intraprostatic invasion of the seminal vesicle was found in 2.2% of cases (23 of 1050) and was not associated with risk of recurrence. This is in line with previous studies reporting that the prognosis of men with intraprostatic SVI is not as poor as that of men with extraprostatic SVI (66). Our finding supports the recommendation issued by the 2009 ISUP Consensus Conference that tumor invasion of the muscle wall of the extraprostatic part of the seminal vesicles is required for stage pT3b (63).

We found that invasion of the seminal vesicle mucosa was associated with a poor clinical outcome (HR 4.2, 95% CI 1.2-14.2, $p=0.021$) (Figure 11) and only three of 19 men with muscle wall invasion alone had s-PSA relapse. To our knowledge, this has never previously been reported. In line with the discussion above, it could be argued that the mechanism behind the poor outcome for men with mucosal invasion is related to certain characteristics required for the infiltration of this structure. Mucosal infiltration was seen in 41 out of 60 SVI cases (68.3%), and was always accompanied by muscle wall invasion. Thus, when reading seminal vesicle slides focus should be on examining the muscular wall. This is helpful for the pathologist since efforts on thoroughly looking for small cancer deposits in the mucosa seem unnecessary.

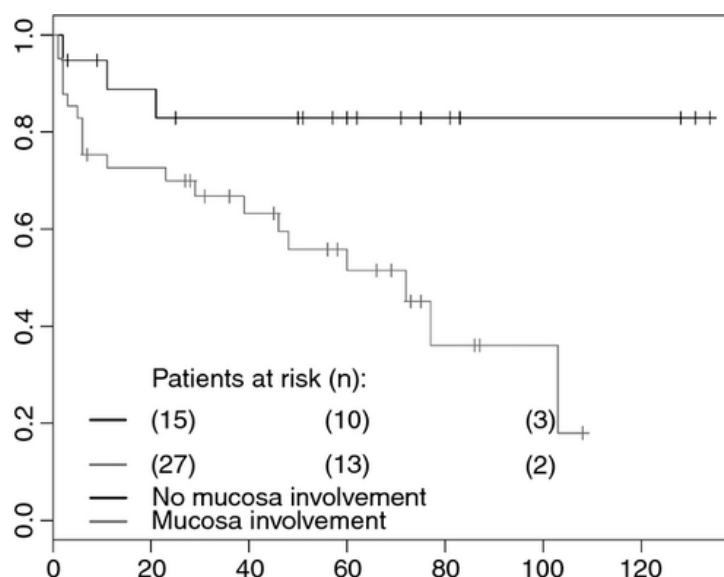


Figure 11. Kaplan-Meier estimates of recurrence-free survival stratified by presence or absence of mucosa invasion among patients with SVI. HR 4.2 (95% CI 1.2–14.2), $p = 0.021$.

Apart from mucosa invasion, neither of the other features evaluated correlated to prognosis. Among the routes of invasion, we found Type 1 (83.3%) to be the most common, while Type 3 was only seen in two cases. The uneven distribution of invasion routes precluded statistical analysis of their prognostic impact. However, the two Type 3 cases did not invade the seminal vesicle mucosa and none of them recurred.

4.1.2 Definition of mucosa

There is no generally acknowledged definition of the mucosa. However, in most cases there is a fairly well-defined muscle wall in the outer layers of the seminal vesicle, contrasting against the mixture of connective tissue and some smooth muscle strands seen between the lobules of mucosal glands. In our study, invasion of the mucosa was defined as cancer infiltrating between tubular seminal vesicle glands, i.e. above a line touching the base of the glandular lobules. Invasion within the epithelium was not required for diagnosis of mucosa invasion and would rather be defined as pagetoid spread. As the boundary between mucosa and muscle wall sometimes is poorly defined, the introduction of routine reporting of mucosal invasion of SVI in clinical practice may need to be preceded by reproducibility studies.

4.1.3 Limitations

There is always a risk of misleading results in a subgroup analysis with small number of cases. However, the number of cases with SVI evaluated in this study is high compared to other contemporary series. As the diagnostic process of prostate cancer has developed, the patients are usually diagnosed at an earlier stage and therefore it is difficult to collect large series with adverse pathological features such as SVI. Furthermore, analysis of the prognostic difference between presence versus absence of seminal vesicle mucosa invasion is a dichotomous split of the SVI group in reasonably large subgroups. Nevertheless, more studies from other centers are needed to validate the results before including mucosal invasion in reporting guidelines.

Early in the study, the entire seminal vesicles were routinely removed at surgery, but later the operation technique was changed to obtain shorter operation time and decreased risk of nerve damage. Therefore, the seminal vesicles were resected completely in 19 cases and partially in 41 cases, in which the tips of the seminal vesicles were not removed. This may have influenced the results of our study since it is difficult to analyze route of invasion in cases with partial resected seminal vesicles. However, the base of the seminal vesicle was always removed and since Type 3 invasion is a rare finding this is not likely to influence the results of the study. Also, the main finding in this study was the prognostic significance of mucosal invasion, which is unlikely affected by partial or complete resection of the seminal vesicles.

4.2 STUDY II: IDC-P MAY HAVE THE POTENTIAL TO GIVE RISE TO METASTASES

4.2.1 Main findings

In the second study of this thesis the aim was to evaluate the genomic aberrations of 45 regions of a prostatectomy specimen and compare them with the genomic aberrations found in the lymph node metastases. This study is based on another report in which initial profiling of the primary tumor did not closely match the genomic characteristics of the lymph node metastases (151). Therefore, this case was examined much more closely and the primary prostatectomy specimen was divided into 45 morphologically distinct regions. Analysis of genomic breakpoints was done to reconstruct phylogeny compared to the metastatic lesions. Eleven areas were lost during tissue harvesting, library preparation or quality control of the data, and another nine foci contained less than five BPRs and were excluded. In total, 25 areas were successfully analyzed. In total, 385 BPRs were found in two or more tumor areas and used to construct the phylogenetic tree.

Interestingly, we found that the areas somatically most closely related to the lymph node metastases were three areas with IDC-P. We verified the diagnosis using immunohistochemistry for p63 and alpha-methylacyl-CoA-racemase (AMACR). The areas with IDC-P had either a complete or fragmented basal cell layer, which confirms the morphological diagnosis of IDC-P (Figure 12).

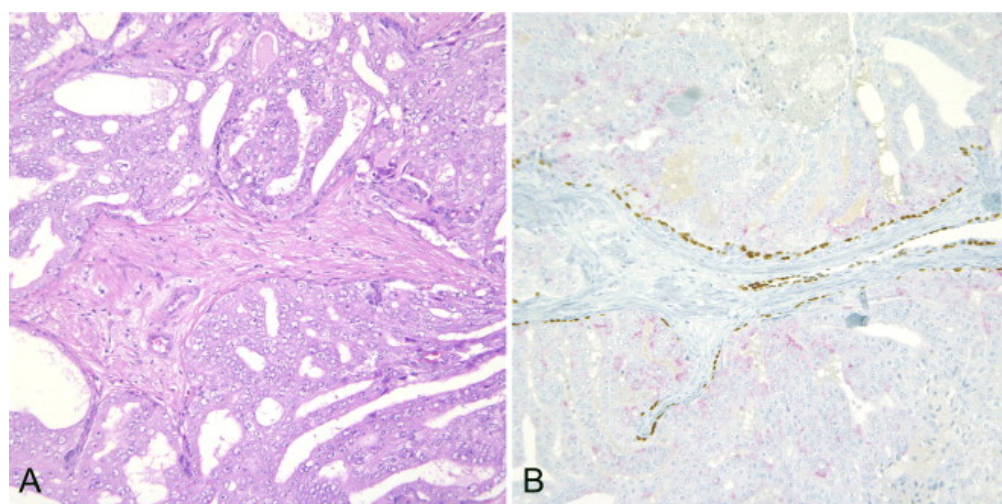


Figure 12. Histological slides of IDC-P (area 8 in the peripheral zone). (A) H&E staining, 20X lens magnification. (B) The same area with immunohistochemical staining for p63 (brown) and AMACR (red), 20X lens magnification.

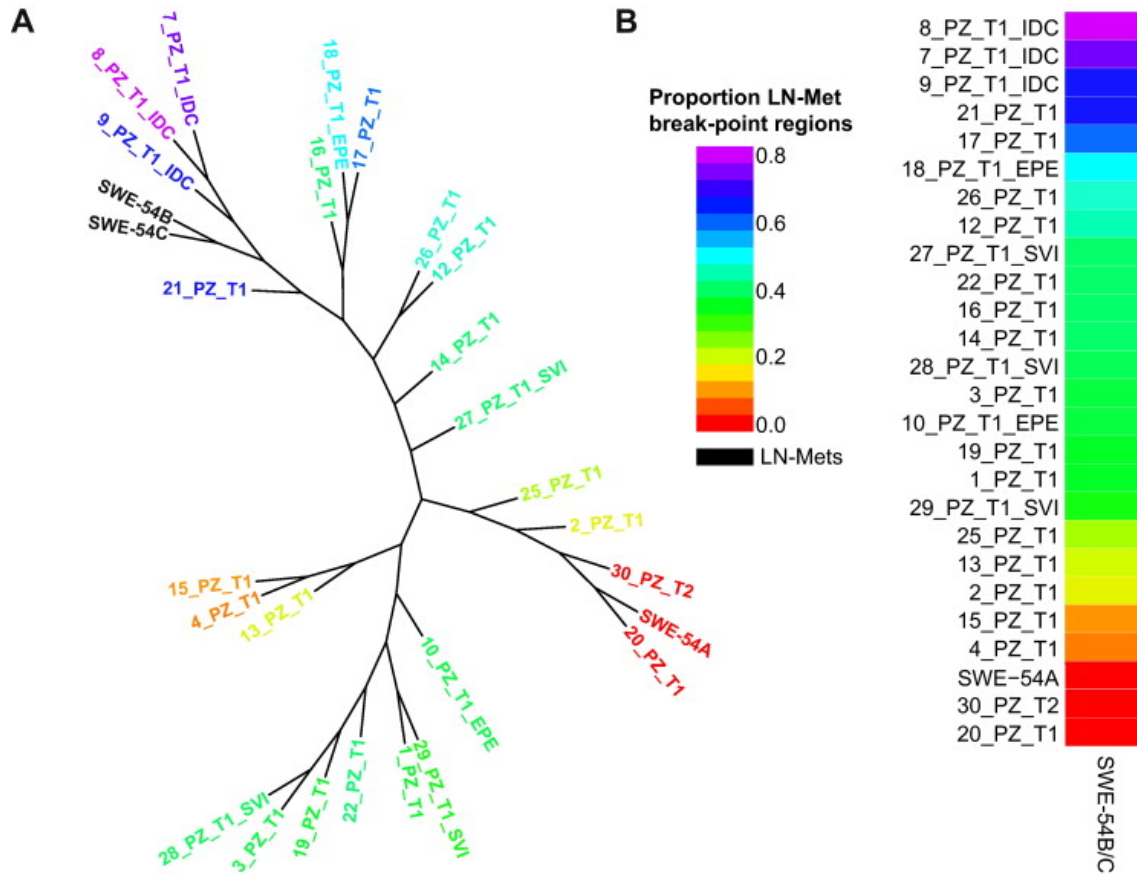


Figure 13. (A) Phylogenetic tree showing the somatic similarity between the tumor areas and the metastases. (B) Samples were colored according to the proportion of BPRs shared with the lymph-node metastases. SWE-54A (primary fresh-frozen tumor sample), SWE-54B (right fresh-frozen lymph-node metastasis) and SWE-54C (left fresh-frozen lymph-node metastasis) represent the tissues previously profiled. Tumor regions are annotated according to area, zone, focus and in some cases additional label. TZ = transition zone; PZ = peripheral zone; IDC = intraductal carcinoma; EPE = extraprostatic extension; SVI = seminal vesicle invasion.

As seen in the phylogenetic tree, another area (21_PZ_T1) was also closely related to the metastases and the areas with IDC-P (Figure 13). This focus was located at some distance (Figure 14) but showed perineural invasion. Area 17 also showed a relatively high degree of similarity with the IDC-P components and this area was located between area 21 and the IDC-P areas (Figure 14). One possible explanation is that the tumor cells of area 17 gained the ability to spread throughout the ducts and along the nerves and became aggressive by this mechanism. These features may have enabled the tumor to colonize other part of the prostate and spread to lymph nodes. As reported previously (151), the fresh-frozen tumor sample from the primary tumor, annotated SWE-54A, did not share any somatic denominator with the metastases. However, in this study we found that one of the analyzed areas (20_PZ_T1) had a high degree of overlap in BPRs with SWE-54A and shared 83% of its BPRs (Figure 15). This

is not surprising as SWE-54A was macroscopically sampled from the same region as the tumor area located in the posterior part of the PZ.

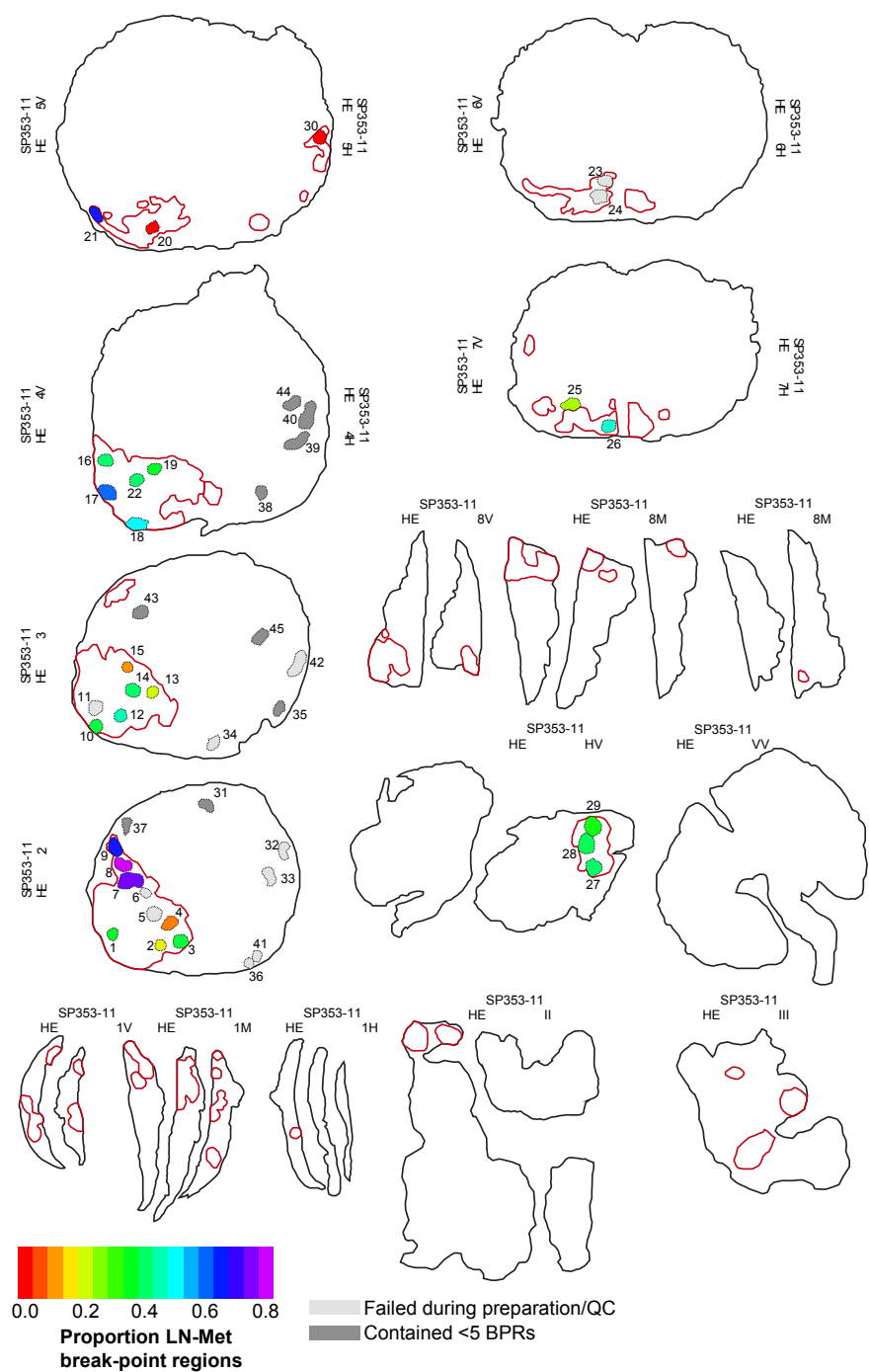


Figure 14. Overview of the whole-mount sections and somatic similarity to the metastases for each area. Description of each area is available in Material and Methods (Table 2). Areas that were lost during sample preparation or quality control are colored light grey. Areas that contained fewer than 5 BPRs were not included in the phylogenetic analysis and colored dark grey.

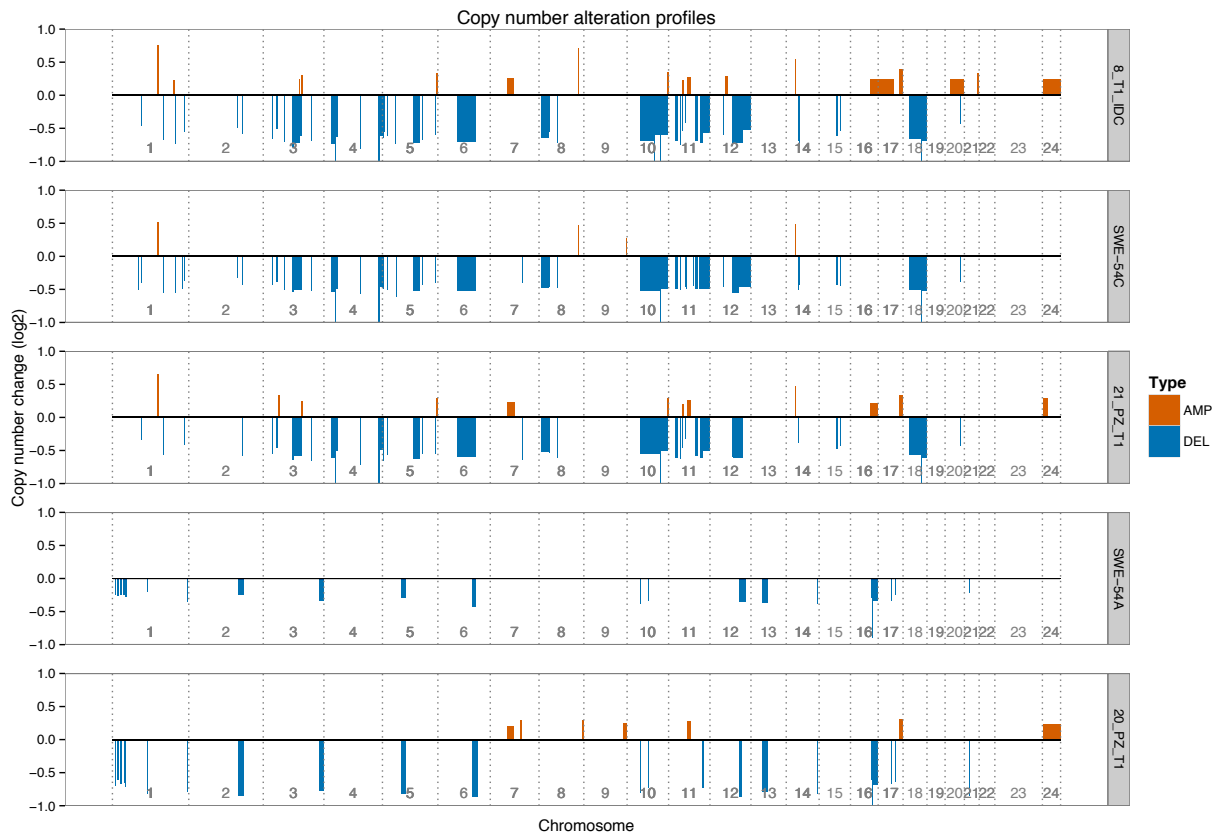


Figure 15. Overview of the copy-number variations for 8_T1_IDC (intraductal carcinoma), SWE-54C (lymph-node metastasis), 21_PZ_T1 (perineural invasion), SWE-54A (previously profiled tissue), and 20_PZ_T1 (highly related to SWE-54A). The three areas in top of the figure show a high degree of somatic similarity.

4.2.2 Genomic evidence for IDC-P as an adverse prognostic factor

IDC-P is commonly associated with adverse prognostic findings on RP such as high GS, high volume and high stage. Also, it is an independent prognostic factor of clinical outcome (44, 156). It has been demonstrated that IDC-P in diagnostic biopsies is strongly associated with metastatic disease after external beam RT (157).

A few studies have been carried out on the molecular background of IDC-P. One study evaluated loss of heterozygosity (LOH) using 12 polymorphic microsatellite markers frequently lost in prostate cancer. They found that LOH was present in 60% of IDC-P, whereas it was seen in 29% of Gleason pattern 4 cancers and absent in Gleason grade 3 cancers (158). In a study by Lotan *et al* cytoplasmic PTEN loss was identified in 84% of IDC-P and none of the cases with high-grade PIN (159). As LOH is a marker of allelic instability and *PTEN* loss is associated with aggressive tumor features both studies provide plausible molecular explanation for why IDC-P is associated with poor clinical outcome.

Although a few studies have shown certain molecular traits of IDC-P that may be correlated to poor prognosis, our study is the first to show that this lesion actually has metastatic seeding potential. This finding supports previous studies on IDC-P as an indicator of adverse prognosis, but also indicates that IDC-P should be reported when found in the RP specimen.

4.2.3 Reporting of IDC-P

As IDC-P has been shown to correlate with aggressive tumors it should always be reported when found on diagnostic biopsies, especially when seen without invasive cancer or with only low-grade cancer (160). In these cases the low-grade tumor is likely not representative of the overall tumor grade. Reporting of IDC-P is probably most important in pre-treatment biopsies. Our finding that metastases were somatically most similar to the IDC-P component suggests a metastatic capacity. It is possible that tumors with an IDC-P component should be treated more aggressively. Therefore, it is important that pathologists recognize and report this feature correctly. Several diagnostic criteria have been suggested for IDC-P. Guo *et al* reported the most commonly used in 2006 (43). According to this study IDC-P should be defined as lumen-spanning intraductal proliferation of atypical epithelial cells with partly or completely preserved basal cell layer in combination with either: (1) solid or dense cribriform patterns or (2) loose cribriform or micropapillary patterns with either marked nuclear atypia (nuclear size 6x normal or larger) or comedonecrosis. This definition is, however, rather unclear. The authors did not justify the 6x cut-off for nuclear enlargement and did not specify whether the nuclear area or diameter should be considered when determining the size. This has led to considerable confusion among uropathologists (42). As a consequence the interobserver reproducibility of IDC-P is low, even among experts in uropathology (161).

4.2.4 Somatic heterogeneity

This study clearly demonstrates the problems with bulk sampling of prostate tumors when mapping the clonality of metastatic disease. A previous study from our group failed to establish a somatic relationship between the primary tumor and metastases, possibly because of the sampling technique (151). Many studies have shown that there is an enormous heterogeneity both between tumor foci and within a single tumor focus of the prostate (134, 162). These studies clearly demonstrate the importance of a more sophisticated sampling. In this study we show that it is possible to identify the metastasizing tumor clone if the samples are microdissected. This is important both for mapping of the metastatic pattern of the tumor and for identification of aggressive clones.

4.3 STUDY III: STAGE PT3B PROSTATE CANCER HAS A POOR PROGNOSIS AND A HIGH TUMOR BURDEN ON PREOPERATIVE NEEDLE BIOPSIES COMPARED TO PT3A CANCER

4.3.1 Main findings

In Study III we retrieved information from the NPCR for the evaluation of the clinical outcome of men with stage pT3b compared to men with stage pT3a after RP. We did this to examine the prognostic significance of the seminal vesicle component among tumors extending beyond the boundaries of the prostate. In addition, we analyzed preoperative biopsy data to correlate biopsy pathology with SVI and EPE. In total, we evaluated 31,415 patients diagnosed between 2000 and 2012 and treated with RP. Out of these patients, 4063 (12.9%) and 1371 (4.4%) were staged as pT3a and pT3b, respectively. In 1163 patients no distinction was made between pT3a and pT3b when registered and these tumors were therefore listed as stage pT3 only. Patients with stage pT3b tumor had in general higher s-PSA at diagnosis and higher GS at RP (Table 3). They also were more likely to receive adjuvant RT and/or ADT.

Table 3. Clinical and pathological features of patients with stage pT3a and pT3b

	pT3a (n=4063)	pT3b (n=1371)	P value
Median follow-up time, RT, years (IQR)	5.3 (3.4-9.7)	5.0 (3.2-9.0)	0.006*
Median follow-up time, ADT, years (IQR)	5.6 (3.5-10.1)	5.4 (3.2-9.7)	0.073*
Median follow-up time, death, years (IQR)	5.7 (3.7-10.2)	5.4 (3.5-9.9)	0.047*
Median s-PSA at diagnosis, ng/ml (IQR)	7.4 (5.3-11.0)	9.2 (6.2-14.0)	<0.001**
Year of diagnosis, n(%)			<0.001***
2000-2003	875 (21.5)	276 (20.1)	
2004-2006	966 (23.8)	266 (19.4)	
2007-2009	727 (17.9)	288 (21.0)	
2010-2012	1495 (36.8)	541 (39.5)	
Median age at RP, years (IQR)	64 (60-67)	65 (61-68)	<0.001**
Age at RP, years, n (%)			<0.001***
<55	302 (7.4)	79 (5.8)	
55-59	703 (17.3)	183 (13.3)	
60-64	1202 (29.6)	377 (27.5)	
65-69	1390 (34.2)	508 (37.1)	
≥70	466 (11.5)	224 (16.3)	
RP-GS, n (%)			<0.001***
2-6	1001 (24.6)	103 (7.5)	
3+4	1636 (40.3)	384 (28.0)	
4+3	899 (22.1)	418 (30.5)	
7	1 (0)	0 (0)	
8	301 (7.4)	212 (15)	
9-10	154 (3.8)	226 (15.5)	
Missing	71 (1.7)	28 (2.0)	
Adjuvant RT, n (%)			<0.001***
Yes	806 (19.8)	455 (33.2)	
No	3257 (80.2)	916 (66.8)	
Adjuvant ADT, n (%)			<0.001***
Yes	154 (3.8)	159 (11.6)	
No	3909 (96.2)	1212 (88.4)	

*Log-Rank test, **Mann-Whitney U test, ***Chi square test. RT, Radiotherapy; ADT, Androgen deprivation therapy; RP, Radical prostatectomy; RP-GS, Radical prostatectomy-Gleason score; s-PSA, serum prostate specific antigen; IQR, Interquartile range.

In multivariate analysis, adjusting for year of diagnosis, age, biopsy grade and s-PSA, patients with stage pT3b had a higher risk of death of prostate cancer (HR 2.3, 95% CI 1.5–3.3, $p<0.001$) and death from any cause (HR 1.5, 95% CI 1.2-1.8, $p<0.001$) compared to patients with stage pT3a (Table 4) (Figure 16). Excluding patients who received adjuvant treatment, they were also more commonly treated with post-operative RT or ADT, indicating clinical recurrence. Although patients with stage pT3b tumors had a higher risk of clinical recurrence, the disease specific survival of this patient group was surprisingly high (Table 5). After six years, prostate cancer specific survival of patients with pT3a and pT3b tumors was 98% and 94%, respectively.

Table 4. Clinical outcome patients with stage pT3a and pT3b

Endpoint	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
All-cause mortality		
pT3a	1.0 (Ref.)	1.0 (Ref.)
pT3b	1.8 (1.5-2.1)	1.5 (1.2-1.8)
PCa-specific mortality		
pT3a	1.0 (Ref.)	1.0 (Ref.)
pT3b	3.0 (2.1-4.2)	2.3 (1.5-3.3)
Treatment-free survival (RT)		
pT3a	1.0 (Ref.)	1.0 (Ref.)
pT3b	1.4 (1.2-1.7)	1.5 (1.4-1.7)
Treatment-free survival (ADT)		
pT3a	1.0 (Ref.)	1.0 (Ref.)
pT3b	3.5 (2.9-4.3)	3.0 (2.5-3.7)

Cox regression models evaluating prognosis of men with pT3b compared with men with pT3a. All comparisons: $p<0.001$. Adjusted: Adjusted for age at RP, year of diagnosis, biopsy GS and s-PSA. RT, Radiotherapy; ADT, Androgen deprivation therapy.

Table 5. Prostate cancer specific survival for patients with stage pT3a and pT3b

Year of radical prostatectomy	Time since radical prostatectomy											
	One year		Two years		Three years		Four years		Five years		Six years	
	pT3a	pT3b	pT3a	pT3b	pT3a	pT3b	pT3a	pT3b	pT3a	pT3b	pT3a	pT3b
2000	100	100	100	100	100	100	99	98	99	96	99	96
2001	100	99	100	99	100	99	99	94	99	94	98	93
2002	100	100	100	99	99	97	99	97	99	96	99	96
2003	100	100	99	100	99	98	99	96	98	94	98	92
2004	100	99	99	99	98	98	98	97	98	96	98	94
2005	100	100	100	100	99	97	99	97	99	96	99	93
2006	100	100	99	99	99	97	98	97	98	96	97	95
2007	100	99	100	99	99	97	99	97	98	96	98	93
2008	99	100	99	99	98	98	97	97	97	97	97	94
2009	100	99	100	98	100	97	99	96	99	94		
2010	100	100	99	98	99	96	99	95				
2011	100	100	100	100	100	99						
2012	100	100	99	100								
All years	100	100	99	99	99	98	99	97	98	96	98	94

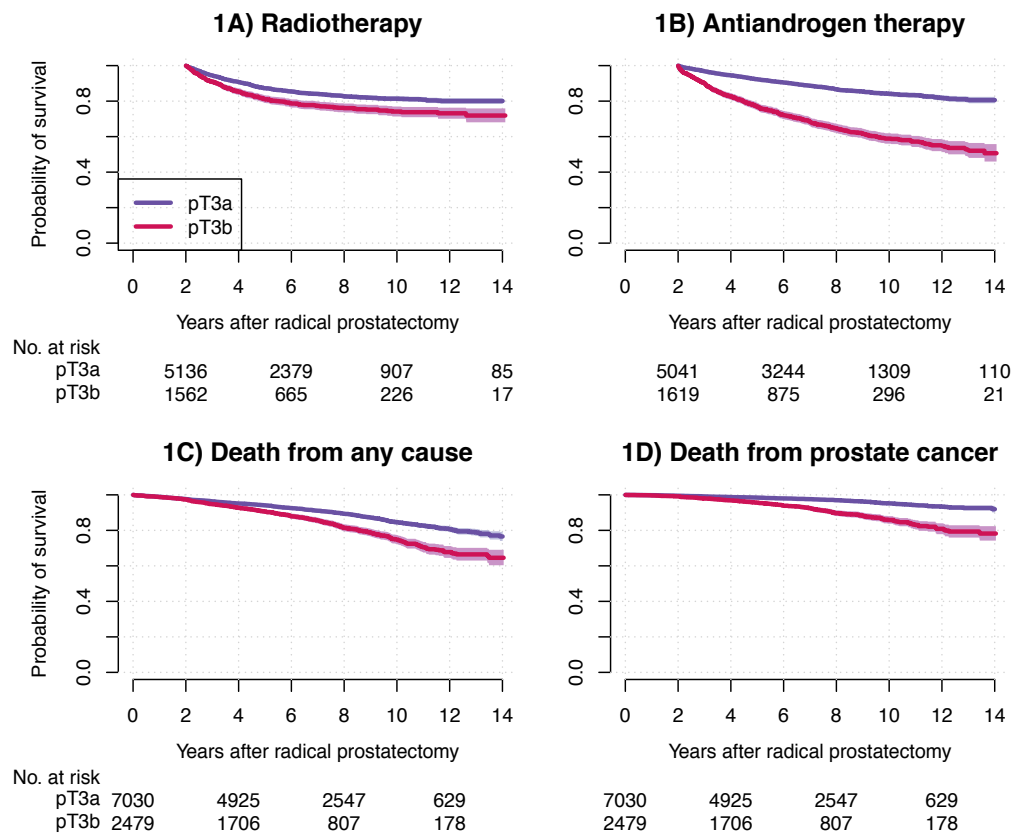


Figure 16. Kaplan-Meier curves showing the survival, by endpoint, between patients with stage pT3a and pT3b tumors. (A) Initiation of postoperative radiotherapy. (B) Initiation of androgen deprivation therapy. (C) Death from any cause. (D) Death from prostate cancer.

In this study we also evaluated the preoperative biopsy findings among patients with stage pT3a vs. pT3b. This was done to see if there were any significant differences in preoperative findings between the two stage categories. We found that patients with pT3b tumors had a greater tumor burden in their biopsies, as analyzed by total cancer length, greater number of positive cores and higher percentage of GS 8-10 (Table 6).

Table 6. Biopsy characteristics of pT3a, pT3b and all pT3

	pT3a (n=1979)	pT3b (n=715)	pT3 (n=372)	P value
Median linear cancer extent, mm (IQR)	14 (6-27)	24 (11-45)	15 (6-32)	<0.001*
Median number of positive biopsies (IQR)	4 (2-6)	5 (3-7)	4 (2-6)	<0.001*
GS at diagnosis, n (%)				
2-6	602 (30)	96 (13)	125 (34)	<0.001**
3+4	794 (40)	240 (34)	123 (33)	
4+3	350 (18)	183 (26)	74 (20)	
8	164 (8)	112 (16)	31 (8)	
9-10	65 (3)	83 (12)	16 (4)	
Missing	4 (0)	1 (0)	3 (1)	

* Mann-Whitney U test; ** Chi square test

4.3.2 General discussion

In this study we confirmed that patients with pT3a disease have a more favorable prognosis compared to men with stage pT3b. In both stage categories the cancer cells have acquired the ability to invade periprostatic tissue, which demonstrates their aggressive phenotype. In line with previous literature the invasion of the seminal vesicles was associated with a poorer clinical outcome compared to EPE alone. Palliative doses of RT were excluded in the study and therefore RT as endpoint should be interpreted as a surrogate marker for local recurrences. Both castration therapy and anti-androgens are included in the concept of ADT and the initiation of ADT could be a reflection of both local recurrence and distant metastatic disease. Hormone therapy is usually not combined with salvage RT. However, this is considered for some patients with adverse findings on the RP and relatively high s-PSA. When there is suspicion of disseminated disease, treatment with bicalutamide and/or medical or surgical castration is usually initiated. Our data indicate that patients with SVI have a higher risk of both local recurrences and metastatic disease compared to patients with EPE alone. The findings may also reflect that these patients are more likely to be offered salvage RT, despite the risk that the disease has already spread to other sites.

We excluded patients who received RT and/or ADT within two years after RP as they possible were treated with adjuvant intention. The exclusion of these patients may introduce a selection bias since patients with the most adverse findings (higher s-PSA, higher grade, more extensive positive surgical margins, etc.) are more likely to receive adjuvant or salvage treatment after surgery. Exclusion of this group would make the overall prognosis seem more favorable. On the other hand, including all patients would also be problematic since the addition of adjuvant treatment would be classified as recurrence, leading to an overestimation of clinical recurrences. Both strategies were discussed and we decided to add Kaplan-Meier

curves with all patients included for comparison (Figure 17). This obviously increases the number of events as many patients received adjuvant treatment but also because patients with stage pT3b disease actually have a high risk of early recurrences that are treated with RT and/or ADT. In Study I we found that patients with SVI have a higher risk of recurrence than those without SVI, especially during the first 20 months. Therefore, many patients with stage pT3b with prostate cancer recurrence are probably lost when censoring treatments given the first 2 years after surgery.

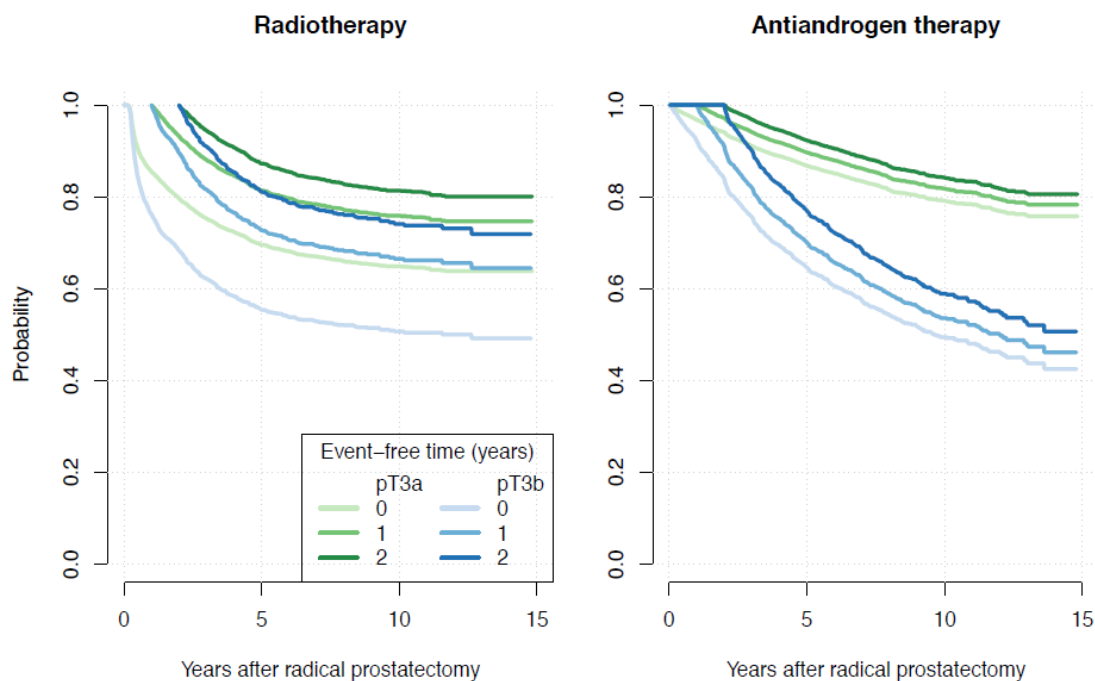
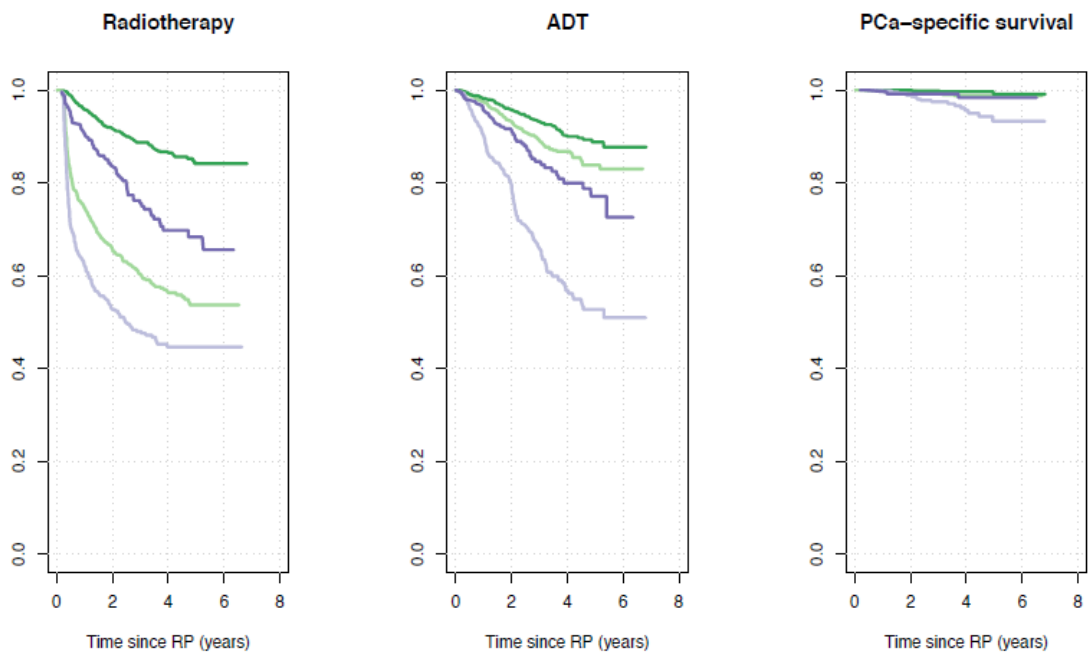


Figure 17. Event-free survival with exclusion of events up to 0, 1 and 2 years after radical prostatectomy.

There are large differences in outcome of the RT graphs when stratifying according to surgical margin status depending on how many years we choose to exclude (Figure 18). A possible explanation is that patients with extensively positive surgical margins and pT3 stage disease are often candidates for early postoperative adjuvant RT. Therefore, RT as an indicator of progression, especially during the first 2 years after surgery, should be interpreted with caution. Obviously, patients with both SVI and positive surgical margins have the highest risk of receiving postoperative treatment and also the highest risk of dying of the disease. This is not surprising as margin positivity itself is known to be an adverse prognostic factor (163). If those who received treatment within 2 years after surgery are not excluded, it becomes apparent that a large proportion of patients with pT3a or pT3b cancer and positive

surgical margins receive postoperative RT. The reason for this may be that surgical margin status plays an important role for the decision on salvage RT or immediate adjuvant RT. Furthermore, we did not consider lymph node status in the analyses. Lymph node metastases are certainly associated with a poorer clinical outcome. However, it is unlikely that adjusting for lymph node status would affect the results significantly since lymph node metastases are uncommon among RP patients nowadays (164).

A



B

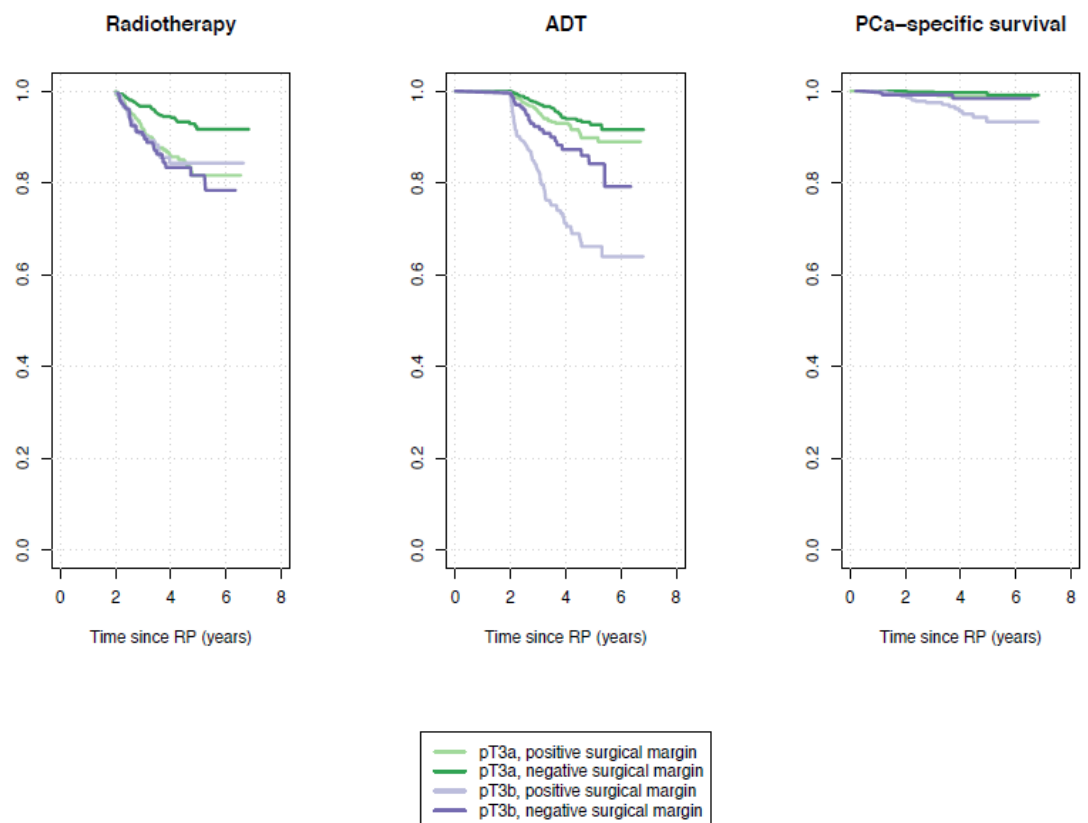


Figure 18. Kaplan–Meier estimates of event-free survival, by endpoint, between patients with staging category pT3a and pT3b tumors stratified according to surgical margin status and exclusion of patients who received treatment (A) 0 and (B) 2 years post-operatively. Year of diagnosis from 2008-2012.

Prostate cancer is often slowly growing and the 6-year disease specific survival was as high as 94% for men with SVI. Improved survival for these patients is probably partly a result of improved treatment options. ADT remains the basis in treatment of advanced prostate cancer, however, docetaxel improve survival for these patients and has now been available in over a decade (165). The last years have offered additional medical treatment as the new second-generation anti-androgens have been introduced to the market. Abiraterone and docetaxel are now approved for treatment of hormone-sensitive prostate cancer, which may further improve survival for patients with advanced prostate cancer. In addition, the RT strategies continuously improve.

Clinical nomograms are commonly used for prediction of pathology prior to surgery. These nomograms usually include s-PSA, biopsy GS and clinical stage and are a basis for treatment planning. In addition, other preoperative biopsy findings such as linear extent and percentage of positive biopsy cores have been suggested as predictors of SVI, however, results are conflicting (89, 91, 101, 102). In our study we found that men with pT3b disease had significantly higher tumor burden in the preoperative biopsies compared to men with pT3a tumors. Our results indicate that the pre-treatment prediction of SVI may improve if total length of cancer involvement of cores and the number of positive biopsies is included in clinical nomograms. However, the interquartile ranges were very broad and the lower quartiles had little tumor burden on biopsies. Therefore, it is doubtful that these data may be of clinical utility. Also, it is likely that improved radiology, with MRI as standard for these patients, add more to the preoperative diagnosis of SVI.

4.3.3 Limitations of registry data

In this study we evaluated the incidence and prognosis of a certain pathological feature based on information from a registry. A limitation of this study design is that no central review using contemporary definitions and grading was done. According to The ISUP consensus conference on Handling and Staging of Radical Prostatectomy Specimens in 2009, SVI should be defined as cancer invading the muscular wall of the extraprostatic part of the seminal vesicles (63). Previously, invasion of tumor cells in the surrounding adipose tissue was sufficient to fulfill the diagnostic criteria of SVI (62). Today, this appearance would rather be staged as pT3a. We had no available information on how SVI was defined in cases reported as pT3b tumors. This may have influenced the incidence and prognosis reported in this study. Furthermore, the Gleason grading system has changed during the last decade with a gradual shift towards higher Gleason grading, particularly after the ISUP 2005 revision (47, 48). Of all patients with pT3b disease in this study, 7.5% had a GS of 2-6. This is not in line

with contemporary RP series in which SVI is an exceedingly rare finding in GS 6 tumors. Recently, 2502 patients with GS 6 at RP were analyzed for EPE and SVI (166). After review according to contemporary evaluation SVI was not found in any of the GS 6 patients and EPE was extremely rare, found in only seven (0.28%) patients. Although these limitations are important to consider, registries are a valuable source of information, which enable analysis of large datasets. This is especially important when analyzing unusual pathological features such as SVI, since clinical cohorts larger than 100 cases would be rare.

4.4 STUDY IV: CONVENTIONAL DIAGNOSTIC PROSTATE BIOPSIES MAY BE AN UNRELIABLE SOURCE FOR PERSONALIZED MEDICINE

4.4.1 Main findings

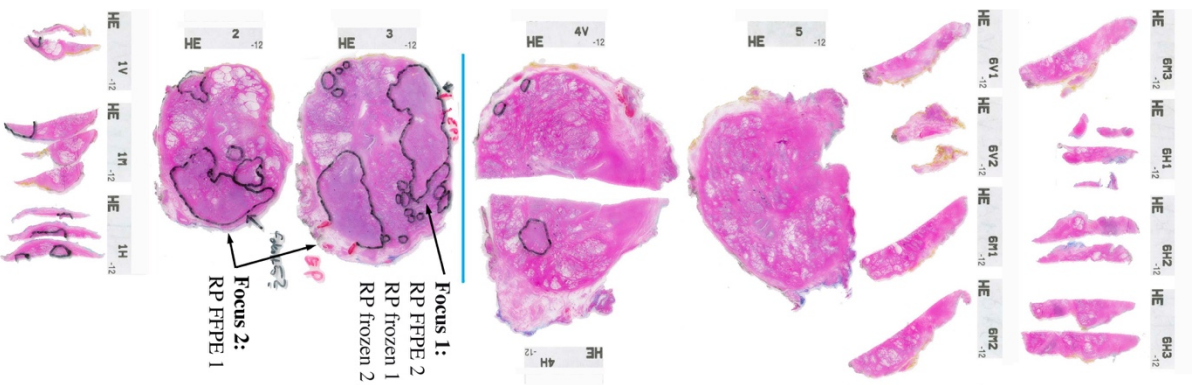
In Study IV the aim was to analyze the genomic heterogeneity of multifocal prostate cancer and to evaluate how well the preoperative biopsies represent the different tumor foci. CNVs, structural rearrangements and point mutations were evaluated in 11 cases of multifocal prostate cancer and corresponding preoperative systematic needle biopsies. In total, 22 biopsies, 25 FFPE and 22 fresh frozen samples from 11 RP specimens were analyzed. Due to low quality of some of the samples we were not able to draw conclusions on somatic origin of 12 prostatectomy samples and seven biopsy samples. Based on the genomic profile of each sample they were annotated “Cancer 1, 2, 3” etc., which refers to the somatically defined tumor and not a spatial tumor focus.

The three tumor foci evaluated in Case 3 all shared a mutation in the *CCND1* gene. This was the only case in which we found a shared mutation between tumor foci. In Case 4 there were no similarity in somatic profile between the fresh frozen sample and the FFPE sample from the same tumor focus. This is an indication of a high degree of intratumoral heterogeneity.

In Case 7 and 11 every sample had high purity and were profiled. In Case 7 we found a distinct separated CNV pattern between the two evaluated tumor foci, indicating that the tumors were somatically independent (Figure 19). The biopsies only carried information from one of the tumor foci, which was located in the right PZ of the prostate. In Case 11 we analyzed two spatially distinct tumor foci, one located in the PZ and the other in the TZ. The two biopsies shared a clonal *SPOP* hotspot mutation with the tumor samples from the tumor in the PZ (Figure 20). The other tumor samples lacked this mutation and the majority of CNVs detected in the PZ tumor. Both biopsies represented the PZ tumor, referred to as Cancer 2.

In summary, 22 somatically independent cancers were detected in 24 tumor foci from 11 patients. Of these, only 10 were represented in the diagnostic biopsies (Figure 21). In eight patients, at least one tumor focus was identified in the biopsies. In only one case the biopsy cores represented two tumor foci, however, in this case a third cancer with a different somatic profile was also identified. Of the mutations and structural variants detected in fresh frozen or FFPE prostatectomy material, only an average of 19% (range 0-44) and 55% (range 0-100), respectively, were found in the preoperative biopsies where a common somatic origin could be established.

A



B

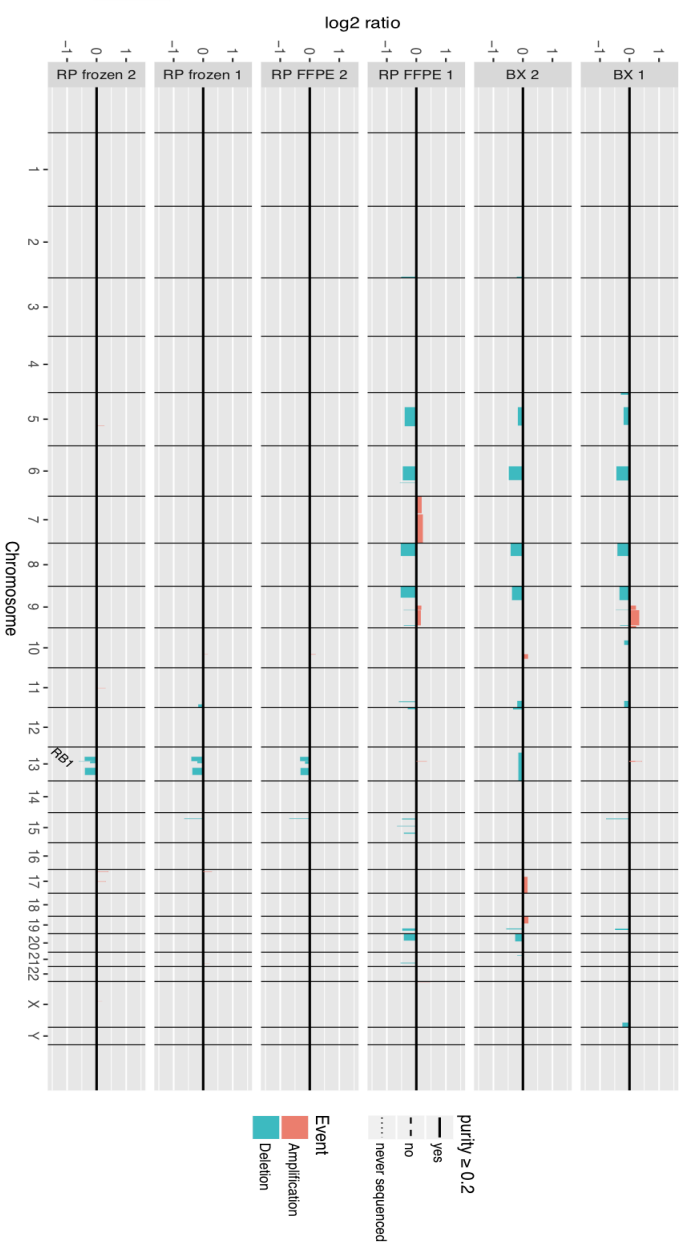


Figure 19. Tumor map and CNV pattern of Case 7. (A) Tumor map showing a large PZ tumor (GS 4+3=7) in the posterior part of the prostate and a bilateral TZ tumor (GS 3+4=7). (B) Tumor samples BX 1, BX 2 and RP FFPE 1 share deletions on chromosome 5, 6, 8 and 9. BX 1 and RP FFPE 1 shared chromosome 9 amplification. RP FFPE 2, RP frozen 1 and RP frozen 2, which are all sampled from the TZ tumor had a different CNV pattern with shared deletions on chromosome 13.

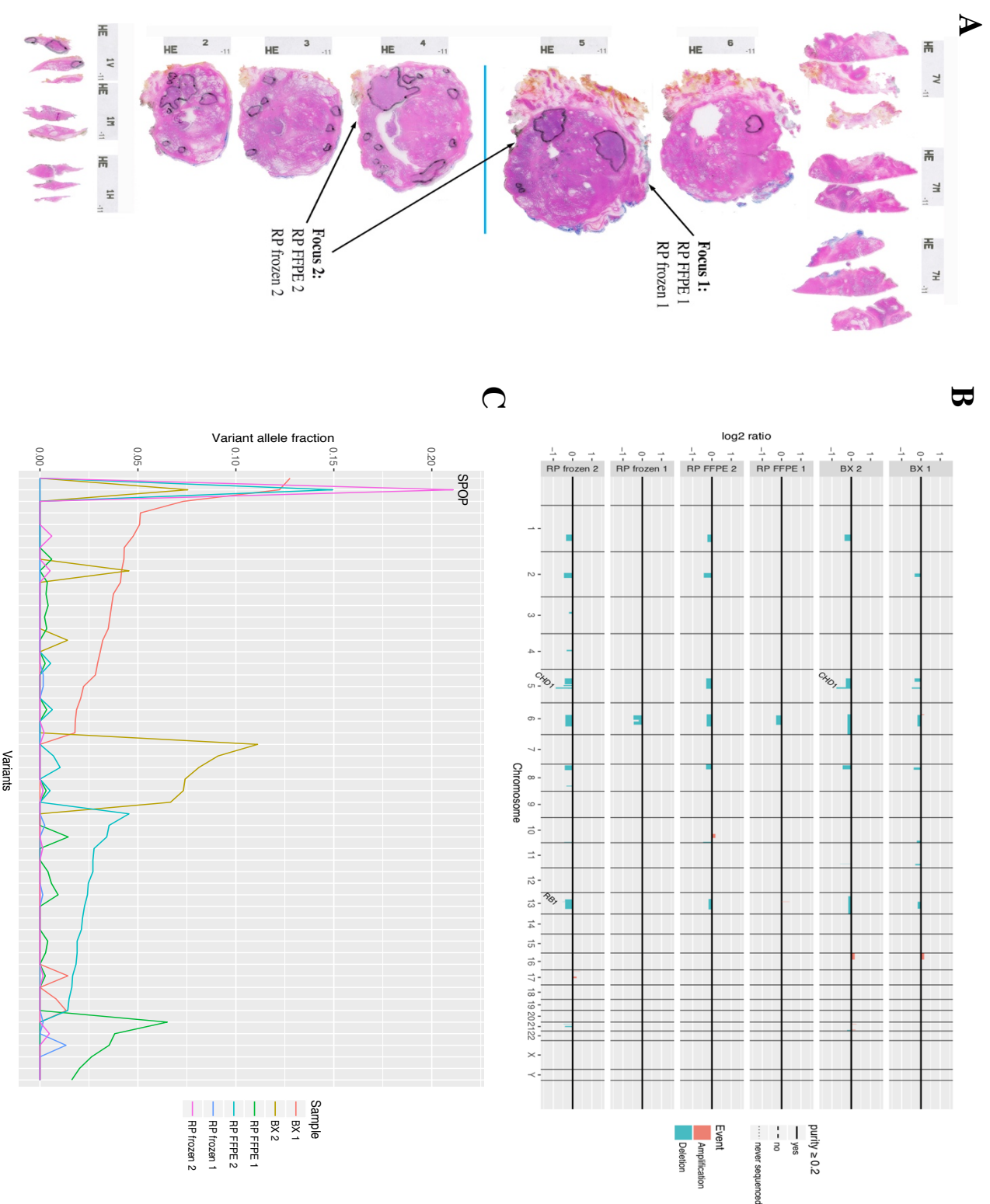


Figure 20. Tumor map and somatic changes of Case 11. (A) Tumor map showing a TZ tumor (GS 3+3=6) in the left part of the prostate and a PZ tumor (GS 4+3=7) in the left posterior part of the prostate. (B) BX 1, BX 2, RP FFPE 2 and RP frozen 2 shared deletion in chromosome 1, 2, 5, 6, 8 and 13, suggesting they belong to the same somatic tumor. (C) The variant allele frequencies of all somatic mutations. X-axis: Individual somatic point mutations and small insertions/deletions. Y-axis: Variant allele frequency. BX 1, BX 2, RP FFPE 2 and RP frozen 2 shared *SPOP* missense mutation.

4.4.2 Somatic heterogeneity

In line with previously published studies (115, 134), our results indicate a high degree of genomic intratumoral heterogeneity. In a recent study, which evaluated 89 tumor foci in 41 men, it was reported that in 76% of cases none of the foci shared any mutations (131). In our study we rarely found any shared aberrations between foci. We identified driver mutations that are important in the pathogenesis of prostate cancer. Mutations in *SPOP*, *PTEN*, *ZMYM3*, *PIK3CA*, *TP53* and *FOXA1* frequently occur in prostate cancer and we found at least one of them in all but one patient. Driver mutations were never shared between foci, but in four patients a driver mutation was shared between biopsy and RP samples. In one case we found mutation in *PIK3CA*, which is frequently mutated in other solid tumors (167) and has been reported as a predictive biomarker for AKT inhibitor (168). However, this mutation was neither found in the biopsy, nor in the other tumor focus. In concordance, Boutros *et al* analyzed a prostate with nine spatially separated foci and found that only two foci had mutations in *PIK3CA* (115). This reflects the risk of missing a potential treatment target when analyzing only parts of the prostate. In a study by VanderWeele *et al* 10 patients with treatment naïve, non-metastatic prostate cancer were examined and 70 spatially distinct regions of the index tumors were analyzed (162). They examined alterations in pathways associated with response in targeted treatment and found that fewer than 25% of mutations were present in all regions of the index tumor. These studies, and the current, are not only evidence of pronounced intratumoral heterogeneity, but also shows how difficult representative tumor sampling is.

4.4.3 Biopsy representation and clinical implications

The GS of diagnostic biopsies is the foundation of the treatment planning process for patients with localized prostate cancer. Both the urologist and the pathologist have important roles in the diagnostic process. From the urologist's point of view, it is important that the needle biopsies actually sample the clinically most important tumor (the tumor with highest grade), while the pathologist has the task of grading the tumor correctly according to current recommendations. But tumors with identical grades may behave differently clinically, which is explained by genetics. Two pathologically identical regions may have significant different somatic profiles. For example, pronounced inter- and intratumoral genomic heterogeneity has been shown to occur in clinically localized GS 7 tumors (115).

Although Gleason grading today is the most important tissue evaluation in prostate cancer, genomic profiling of tumors will be increasingly important in clinical practice. Studies have been carried out to evaluate the effect of neoadjuvant treatment prior to RP, but the clinical

outcome for the patients has not been improved (169). Possibly, genomic profiling of tumor tissue will distinguish patients who may benefit from such treatment. As core biopsy is the tool used at initial assessment, it is important to have a comprehension on how well the biopsy findings correspond to the RP findings. In this study we found that evaluation of a few needle biopsies does not provide a representative picture of somatically separated tumors. However, this is the first study that compared biopsies with RP specimens and therefore more studies are needed in this field.

4.4.4 Limitations

There are a few limitations of this study. In some of the samples there was low tumor purity, which made it hard to draw conclusions on somatic origin. This may be explained by the infiltrating growth pattern often seen in prostate cancer. In comparison to other solid tumors where pure tumor tissue often can be harvested, sampling of prostate cancer is usually more challenging. Moreover, not more than three biopsy cores were analyzed and only cores with more than 2 mm cancer were included. Not including all positive biopsies may have hampered the representation of somatically different tumors. However, an advantage of this approach is that the study design is similar to the clinical practice, where only a limited number of biopsies can be analyzed.

In the study we analyzed biopsies that were sampled systematically. Studies have shown that MRI-targeted biopsies detect more clinically significant cancers (27, 28). Therefore, the biopsies may have been more representative of the tumors if they were targeted. However, it has been shown that even within an index focus there may be somatically different clones (134). This was also confirmed in Study II of this thesis, where we found a high degree of variation in CNVs between small areas within a single tumor focus. Therefore, it is not certain that targeted biopsies towards the index tumor would result in a better mapping of somatically separated tumor clones.

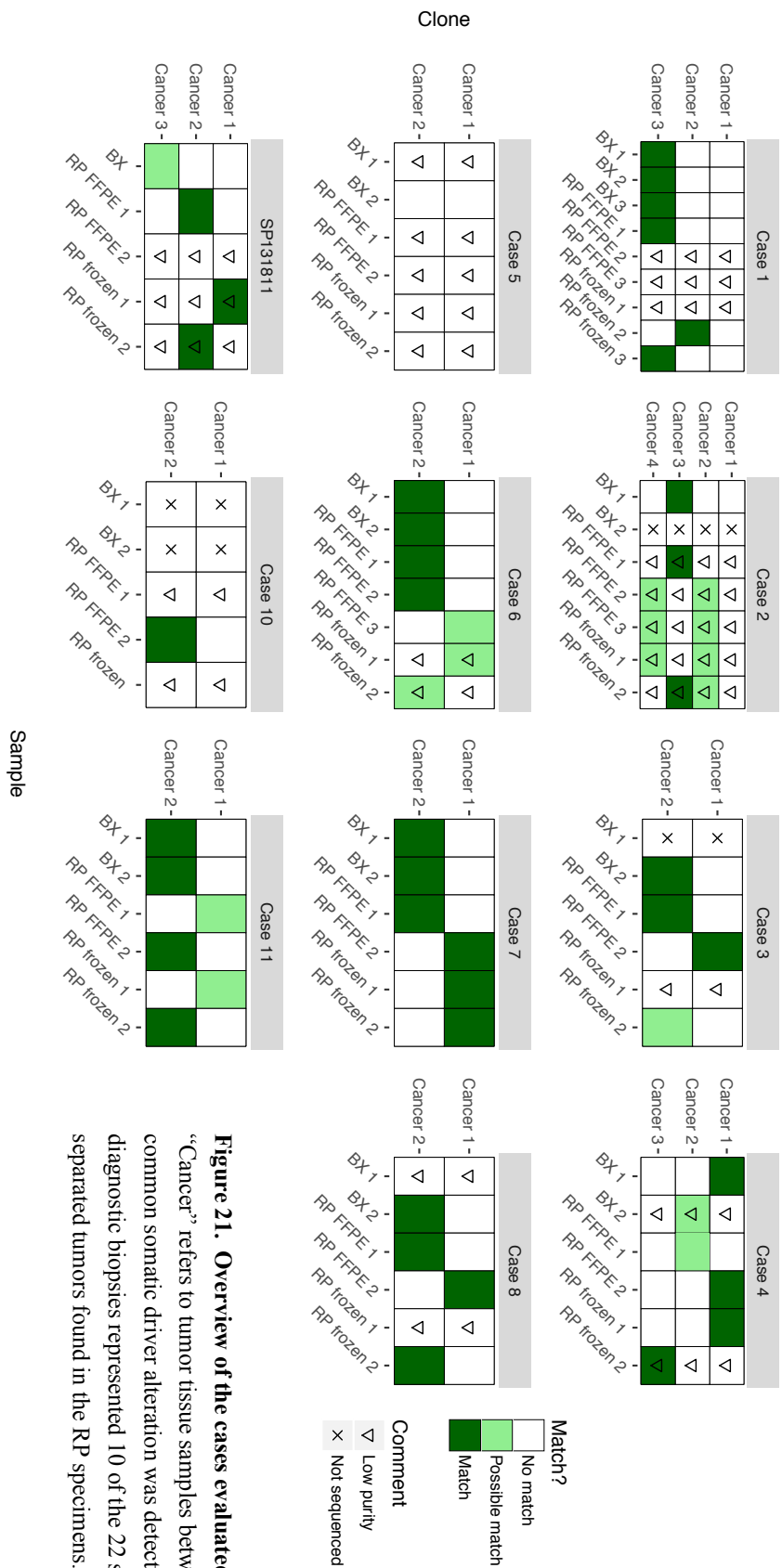


Figure 21. Overview of the cases evaluated in Study IV.
 “Cancer” refers to tumor tissue samples between which a common somatic driver alteration was detected. In total, the diagnostic biopsies represented 10 of the 22 somatically separated tumors found in the RP specimens.

5 CONCLUSIONS

In **Study I** we demonstrated that patients with SVI of prostate cancer have a poorer clinical outcome compared to patients without SVI. However, the prognosis for men with SVI is not uniformly poor. It seems that the prognosis is worse for those with tumor invasion of the seminal vesicles mucosa than for those where SVI is restricted to the muscular wall and connective tissue. Men with SVI without mucosal invasion seem to have a similar outcome as those with no SVI. We found that invasion of the seminal vesicle mucosa was only seen together with invasion of the muscular wall. This is helpful information for the pathologists when reading the slides. Quantitative measures of SVI (area, diameter, uni- versus bilateral involvement) and GS of the SVI component are not useful predictors of recurrence and do not need to be reported by the pathologist.

The IDC-P component in the prostate cancer of **Study II** was somatically very similar to the lymph node metastases of this case, indicating that IDC-P may have metastatic potential. IDC-P is known to be an adverse pathological feature associated with poor clinical outcome. The current study is the first to report a somatic profile of IDC-P areas within a prostate cancer and correlate it with the genomics of metastases. Bulk sampling of the primary tumor was insufficient for establishing its relationship with the metastases. The clone that had seeded the metastases was not found until several distinct tumor areas of the main tumor were microdissected and analyzed. This reflects the pronounced tumor heterogeneity of prostate cancer, which is important to consider in studies analyzing the origin and metastatic pattern of prostate cancer. The results of this study also emphasize the importance of reporting IDC-P when found either associated with invasive cancer or in isolation.

In **Study III** we found that SVI is associated with a poorer clinical outcome, defined as higher risk of initiation of post-operative RT and/or ADT and higher risk of death from prostate cancer, compared to EPE alone. Cancers with EPE or SVI have demonstrated their potential to spread outside of the prostate. The mechanism behind the poorer outcome for patients with SVI remains unknown. Patients with pT3b cancer seem to have a higher tumor burden in preoperative biopsies than those with pT3a. However, the cancer involvement in biopsies varies widely within each stage, which makes it unlikely that this finding will have much clinical utility.

In **Study IV** we found that only some of somatically distinct tumors could be identified in multiple diagnostic biopsies. We also found an enormous genomic heterogeneity, not only between tumor foci, but also within a single focus. This has implications for tumor sampling,

which needs to be done thoroughly to enable identification of somatically different foci that may serve as a basis for treatment planning.

We conclude that prostate cancer has a pronounced heterogeneity of morphology, molecular profile and outcome. Tumors of the same clinical stage may have completely different prognosis due to their unique tumor characteristics. A single case of prostate cancer may have different morphology and genomic profiles in different parts of the prostate and even within tumor foci. This leads to diagnostic challenges in the identification of the clinically most important tumor focus. As the possibilities of oncological treatment increase, it is likely that therapy in the future will be based on the genomics of the tumor. Therefore, it is of uttermost importance that the right clone is being selected for analysis. How this will be done in an optimized manner remains to be studied.

6 FUTURE PERSPECTIVES

Histopathology is an important tool in risk stratification and management of prostate cancer, but personalized genomic profiling will be increasingly important in the future as our understanding of the molecular basis of prostate cancer deepens. This will help to provide the most effective treatment for each patient. Every patient has a unique tumor with a distinct molecular profile. There is also pronounced intratumoral diversity, with different genomic characteristics between primary and metastatic lesions and between tumor foci in the primary tumor (Figure 22). Also, index foci have been reported to be somatically heterogeneous, with many alterations present in subclonal populations (134). Collecting representative tumor samples for identification of relevant tumor clones may therefore be challenging, which furthermore limits the clinical use of genetic biomarkers.

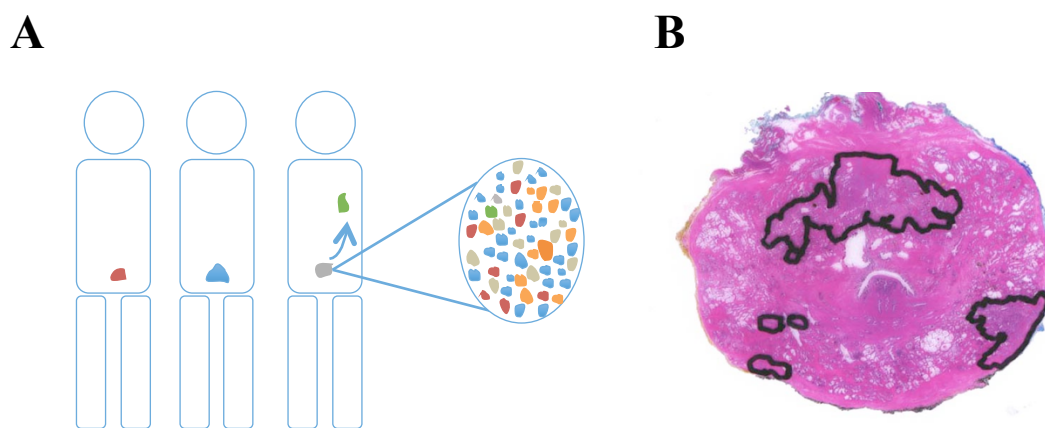


Figure 22. (A) The genomic profile of prostate tumors varies between patients and within a single patient. There is also a intratumoral diversity within a single tumor focus. (B) Multifocal prostate cancer with index focus in the transition zone and smaller foci in the peripheral zone. The somatic profile of the different tumor foci may differ significantly.

There are a few targeted therapies based on genomic changes that have shown promising results in metastatic prostate cancer. Patients who have high expression levels of *AR* usually respond well to *AR* targeted therapies (enzalutamide and abirateron) (170). Patients with germline *BRCA* mutations have recently been shown to have a high response rate to the PARP-inhibitor olaparib (20). *PIK3CA* mutation, which is frequently mutated in prostate cancer and other solid tumors (167), predicts response to AKT inhibitor (168). There are several ongoing clinical trials that further evaluate the effect of PARP and AKT inhibitors in this patient group. However, this thesis and previous studies show that potential treatment targets may be missed when only analyzing limited samples of the cancer.

A better understanding of the pathogenesis of prostate cancer is needed to improve prognostic and predictive biomarkers. A large number of studies have been carried out to evaluate the clonality and heterogeneity of multifocal prostate cancer. A limitation of many studies is that a bulk of tumor tissue has been analyzed. Mutations that are present in a small subset of cells may therefore be diluted and missed. In order to thoroughly analyze the origin and clonality of prostate cancer single-cell analysis should be applied. This has been done in breast cancer and indicates a high level of diversity between cells in a tumor and that no two single cells are somatically identical (171). Analysis on a single cell level provides more information and increases our understanding of the tumor evolution. In addition, it can better predict response and resistance to treatment since a few cells may harbor mutations associated with resistance before treatment is initiated. Only a few authors have used single-cell DNA analysis to examine the somatic heterogeneity of prostate cancer (172, 173). Su *et al* recently studied the genomic heterogeneity at a single cell level in two cases of prostate cancer (173). The cells were collected using LCM. They demonstrated a significant genomic heterogeneity between cells from different parts of the prostate. In the first case they found the same *TP53* driver mutation among all cells. But in the second case they found *TP53* and a few other mutated oncogenes only in some of the cells. They concluded that the findings were consistent with a monoclonal origin of prostate cancer in the first case and a polyclonal origin in the second case. The report does not reveal how many cells that were collected for analysis. Using LCM in single cell analysis of prostate tissue is not reliable if only a single cell is microdissected since the sampling is often done on thin sections, often resulting in only a proportion of a cell being sampled.

Single-cell analysis may also become important in the diagnostic situation. In a recently published study, single cell nuclei analysis from prostate core biopsies of 11 patients was done (172). CNVs were analyzed to examine the heterogeneity and clonal relationships between subpopulations. Cells were sampled through gentle washing of the biopsy core before formalin fixation. The biopsies that contained cancer according to the histopathology examination usually had cells with shared copy-number events, which was interpreted as evidence for clonal expansion. Such clones were typically not seen in benign tissue and the authors concluded that genomic analysis on single cell basis may serve as a tool for prediction of postoperative pathology.

Certainly, more studies in this field are needed to increase the understanding of how prostate cancer evolves and how it can be interpreted to improve diagnostics and treatments. Genomic alterations could be the key to define patients with aggressive disease and ability to respond

to certain treatments. The somatic heterogeneity of prostate cancer aggravates the introduction of genomic profiling into clinical use. For tailoring of treatment it is of great importance not only to evaluate the genomic profile of prostate cancer and identify clones of particular aggressiveness, but also to further evaluate the heterogeneity of the tumors and potential sampling issues in the clinical setting.

7 ACKNOWLEDGEMENTS

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